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Fatal Attraction:

NK cell migration toward and activity in solid tumors

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Stockholm 2018

The cover was designed by my friend and artist Tim Bohlender (www.timbohlender.de), and symbolizes to me the chemokine gradient pulling NK cells to the tumor.

Published by Karolinska Institutet.
Printed by Eprint AB 2018, Stockholm, Sweden.

ISBN 978-91-7676-997-3

Fatal Attraction:
NK cell migration toward and activity in solid tumors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Will be publicly defended in Radiumhemmet's lecture hall, P1:01,
Karolinska University Hospital, Stockholm

Friday, the 6th April 2018, at 10:00

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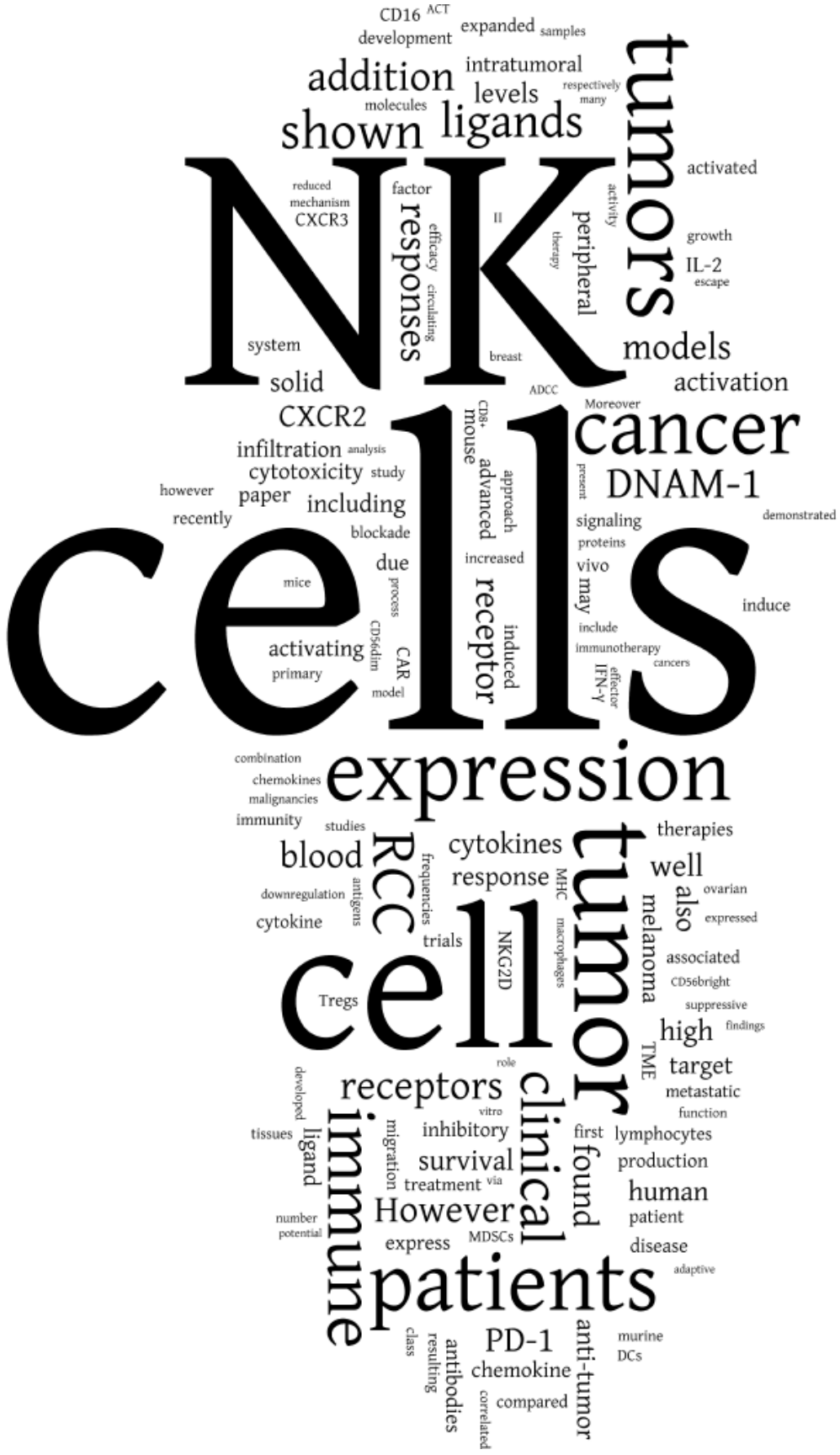
To my parents and Vincent

We cannot direct the wind, but we can adjust the sails.

– Proverb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ABSTRACT

Natural killer (NK) cells play a key role in tumor immunosurveillance due to their ability to induce apoptosis of tumor cells and produce pro-inflammatory cytokines, without prior immune sensitization. In particular, NK cells demonstrate potent anti-tumor immune responses against metastases and hematological malignancies. These characteristics make NK cells attractive for cancer immunotherapy. In contrast to hematological malignancies, however, adoptive transfer of NK cells has so far not provided clinical benefit in patients with solid tumors. Critical barriers in targeting solid tumors include inefficient homing of infused cells to tumor sites and immunosuppression in the tumor microenvironment. In this thesis, I have investigated strategies to improve NK cell migration to solid tumors and explored the immune landscape in patients with renal cell carcinoma (RCC), focusing on NK cells.

In the first part of this thesis, local production of ligands for the chemokine receptor CXCR3 was induced in the microenvironment of melanoma tumors, which enhanced intratumoral localization of infused *ex vivo* expanded human NK cells in mouse xenograft models, resulting in superior anti-tumor immunity (**paper I**). Subsequently, we genetically engineered human NK cells to express the chemokine receptor CXCR2, which conferred them with the ability to specifically migrate to recombinant and RCC tumor-derived CXCR2 ligands, enabling improved targeting of tumor cells *in vitro* (**paper II**).

In the second part, we performed a comprehensive analysis of immune cell and soluble factor profiles in blood and tumor biopsies of 14 patients with primary RCC, identifying factors important for NK cell migration, activation, and immunosuppression (**paper III**). We found profound changes in intratumoral NK cell phenotypes compared with those in peripheral blood, with downregulation of the activation receptor DNAM-1 possibly representing a tumor immune escape mechanism. Moreover, we identified low expression of DNAM-1 and PD-1 on intratumoral and circulating NK cells, respectively, as potential biomarkers of disease progression.

In summary, approaches to improve NK cell homing to tumors and a greater understanding of the RCC immune landscape provided in this thesis, will advance the use and increase the success of NK cell-based therapies in patients with solid tumors.

LIST OF SCIENTIFIC PAPERS

I. Erik Wennerberg, **VERONIKA KREMER**, Richard Childs, Andreas Lundqvist. CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth in vivo. *Cancer Immunology Immunotherapy.* 2015 Feb;64(2):225-35

II. **VERONIKA KREMER**, Maarten A. Ligtenberg, Rosa Zendehdel, Christina Seitz, Annet Duivenvoorden, Erik Wennerberg, Eugenia Colón, Ann-Helén Scherman-Plogell, Andreas Lundqvist. Genetic engineering of human NK cells to express CXCR2 improves migration to renal cell carcinoma. *Journal for ImmunoTherapy of Cancer.* 2017 Sep 19;5(1):73

III. **VERONIKA KREMER**, Christina Seitz, Nicholas P. Tobin, Maarten A. Ligtenberg, Elina Staaf, Jonas Bergh, Evren Alici, Eugenia Colón, Ann-Helén Scherman-Plogell, Andreas Lundqvist. Immune-profiling reveals DNAM-1 downregulation in tumor-infiltrating lymphocytes of renal cell carcinoma patients. *Manuscript.* 2018

CONTENTS

LIST OF ABBREVIATIONS

aAPC	Artificial APC
ACT	Adoptive cell therapy
ADAM	A disintegrin and metalloproteinase
ADCC	Antibody-dependent cellular cytotoxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BAG3	Bcl2-associated athanogene 3
BCR	B cell receptor
CAR	Chimeric antigen receptor
CMV	Cytomegalovirus
CRC	Colorectal cancer
CCL	C-C motif ligand
CCR	C-C motif receptor
ccRCC	Clear cell RCC
COX-2	Cyclooxygenase-2
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
CX3CL	C-X3-C motif ligand
CX3CR	C-X3-C motif receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EBV	Epstein–Barr virus
EGFR	Epidermal growth factor receptor
FasL	Fas ligand
FcγR	Fc receptor for IgG
FcεR	Fc receptor for IgE
FDA	Food and Drug Administration
FoxP3	Forkhead box P3
HCMV	Human CMV
Her2/neu	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor

HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
ILT2	Ig-like transcript 2
IL	Interleukin
ILC	Innate lymphoid progenitor
iNOS	Inducible nitric oxide synthase
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptor
Lag-3	Lymphocyte activation gene-3
LAK	Lymphokine-activated killer
LCL	Lymphoblastoid cell line
LFA	Lymphocyte function-associated antigen
mb	Membrane-bound
MCMV	Murine CMV
MDS	Myeloid dysplastic syndrome
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MIC	MHC class I-related chain
MM	Multiple myeloma
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
M-MDSC	Monocytic MDSC
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NK	Natural killer
NO	Nitric oxide
NOD	Non-obese diabetic
NSCLC	Non-small cell lung cancer
OPLS	Orthogonal Projections to Latent Structures
OPLS-EP	OPLS- Effect Projections
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death 1
PD-L	Programmed death ligand

PGE2	Prostaglandin E2
PMN-MDSC	Polymorphonuclear MDSC
Poly I:C	Polyinosinic:polycytidylic acid
PPR	Pattern recognition receptor
PVR	Poliovirus receptor
rh	Recombinant human
ROS	Reactive oxygen species
RCC	Renal cell carcinoma
scFv	Single chain variable fragment
SCID	Severe combined immunodeficiency
SLT	Secondary lymphoid tissue
S1P	Sphingosine 1-phosphate
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TIGIT	T-Cell Immunoreceptor with Ig and ITIM Domains
TIL	Tumor-infiltrating lymphocyte
Tim-3	T cell Ig and mucin domain-3
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis inducing-ligand
Treg	Regulatory T cell
ULBP	UL16-binding protein
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

1 TUMOR IMMUNOLOGY

1.1 THE IMMUNE SYSTEM IN A NUTSHELL

The immune system fights off pathogens, such as viruses, bacteria, and fungi to protect us from disease. It consists of a complex network of different organs, proteins, and types of white blood cells (leukocytes), the latter having been proposed to be the cornerstone of the immune response by Ilya Mechnikov in 1883 [1]. Developing from hematopoietic stem cells (HSCs), leukocytes commit to either lymphoid or myeloid lineage. The common lymphoid progenitor cell gives rise to plasmacytoid dendritic cells (DCs), B cells, T cells, natural killer (NK) cells, and innate lymphoid cells (ILCs) [2, 3]. The common myeloid progenitor cell differentiates into different types of granulocytes, including neutrophils, eosinophils, basophils, and mast cells, and monocytes that give rise to macrophages and conventional DCs [2]. The immune system is classically divided into innate and adaptive arms, although these boundaries have blurred over recent years with the recognition that some leukocytes display both innate and adaptive features.

1.1.1 Innate immunity

Innate immunity is characterized by a fast response to intruding pathogens and other causes of tissue damage. It is activated by recognition of pathogen-associated molecular patterns and endogenous damage-associated molecular patterns through a plethora of germline-encoded pattern recognition receptors (PRRs) [4]. Signaling through PPRs results in the induction of a broad spectrum of pro-inflammatory mediators, including cytokines, chemokines, and cell adhesion molecules. Together, they orchestrate early host response to infection and alert the adaptive immune system. Activated neutrophils phagocytose and kill bacteria, eosinophils and basophils are involved in protection from parasites, NK cells kill virus-infected cells, and DCs, as professional antigen-presenting cells (APCs), play a central role in bridging innate and adaptive immunity. Upon ingestion of bacteria and viruses, DCs mature, migrate from the site of infection into secondary lymphoid tissues (SLT), proteolytically degrade the pathogens, and display their peptides on major histocompatibility complex (MHC) molecules to T cells [5]. In addition, DCs and NK cells interact with each other via cytokines and cell-cell contact, resulting in mutual regulation [6].

1.1.2 Adaptive immunity

Adaptive immunity relies on a diverse repertoire of highly specific receptors for unique antigens, which is generated through rearrangement of genes encoding different parts of the T cell receptor (TCR) or B cell receptor (BCR). Another hallmark of adaptive immunity is

development of immunological memory that allows for faster and stronger secondary responses. Naïve T cells require three signals to become fully activated and initiate clonal expansion; otherwise they may become anergic or suppressive [2]. The first signal is provided by encounter of a foreign antigen presented by APCs on MHC class I molecules to cytotoxic $CD8^+$ T cells, and on MHC class II molecules to $CD4^+$ T helper cells. Ligation of the CD28 receptor on T cells by co-stimulatory molecules upregulated on DCs, such as CD80 and CD86, gives the second signal. The third signal comes in the form of cytokines either released by plasmacytoid DCs or other cells in the microenvironment [7]. Specific combinations of cytokines direct the differentiation of T cells into subtypes, thereby shaping the quality of the T cell response. For instance, interleukin (IL)-12 induces interferon (IFN)-γ production, the lytic function of $CD8^+$ T cells, and differentiation of $CD4^+$ T cells into a T helper (Th) 1 phenotype [8].

B cells recognize specific cell surface antigens via the BCR, independent of MHC molecules. They process and present antigen to Th2 type $CD4^+$ T cells that stimulate B cells to develop into plasma cells, which produce clonal antigen-specific antibodies or immunoglobulins (Ig) [2]. The cytokines present in the microenvironment determine the Ig isotype that defines the effector function of an antibody via structural differences in the constant Fc region [2]. IgG1 antibodies, for instance, mediate antibody-dependent cellular cytotoxicity (ADCC) of opsonized pathogens and target cells, by immune cells equipped with Fcγ receptors (FcγRs), such as macrophages and NK cells.

1.2 IMMUNITY MEETS CANCER

Cancer is a disease of uncontrollably proliferating cells that can invade other tissues. This unrestricted cell growth is enabled by mutations in proto-oncogenes and tumor suppressor genes arising mostly from errors during DNA replication [9], but also from environmental carcinogens and oncoviruses. Most tumors have two to eight driver mutations that accumulate over decades [10] and, in addition to sustaining proliferation, endow them with resistance to apoptosis, replicative immortality, angiogenesis, insensitivity to growth suppressors, deregulated metabolism, genomic instability, capability for invasion and metastasis, tumor-promoting inflammation, and the ability to evade immune destruction [11].

1.2.1 Anti-tumor immunity

The idea that the immune system may suppress cancer development in the host was first proposed by Ehrlich in 1909 [12] and formed the basis of the 'immune surveillance' hypothesis by Thomas and Burnet in the 1960s. However, is was not until decades later — when suitable

genetic knockout mouse models were developed, demonstrating that carcinogen-induced sarcomas were recognized and rejected by the immune system — that the concept was confirmed [13, 14].

In humans, indirect evidence for tumor immunosurveillance came from higher cancer incidences in immunosuppressed transplant patients than in the general population and from occasional spontaneous tumor regressions [15-17]. Moreover, the presence of lymphocytes within the tumor has been associated with good clinical outcome. Indeed, infiltration of $CD8^+$ T cells has been associated with clinical benefit in a multitude of cancer types, including melanoma, head and neck, breast, ovarian cancer, colorectal carcinoma (CRC), and non-small cell lung cancer (NSCLC) [18]. Galon *et al.* have shown that $CD3^+$ T cells and $CD45RO^+$ memory T cells in the tumor center as well as in the invasive margin independently predict disease-free survival in CRC patients, thereby establishing the immune contexture as a prognostic factor complementary, if not in fact superior, to TNM staging [19]. This concept has been translated into a clinical 'immunoscore' classification system enumerating $CD3^+$ and $CD8^+$ T cells, which is being evaluated in research centers worldwide [20]. NK cell infiltration has also been correlated with good prognosis in a number of cancers (described in more detail in chapter 2.5.1). Conversely, the presence of suppressive immune cells, including M2-skewed tumor-associated macrophages (TAMs) [21], myeloid derived suppressor cells (MDSCs) [22], and regulatory T cells (Tregs) [23, 24], has been shown to predict poor patient outcomes.

The anti-tumor immune response leading to cancer elimination is a multi-step process termed the 'cancer-immunity cycle' [25]. Initially, cells of the innate immune system, including NK cells and macrophages, are alerted and recruited to the tumor, probably due local tissue disruption. In a self-perpetuating loop, M1 macrophages stimulate NK cells to produce IFN-γ that, in turn, activates macrophages to release reactive oxygen species (ROS) and nitric oxide (NO), increases MHC class I expression on tumor cells and inhibits their proliferation [14, 26]. The initial immune attack releases tumor-associated antigens (TAAs) that are ingested by DCs recruited and activated by chemokines and cytokines in the tumor microenvironment (TME). Similar to the process occurring during infection, DCs prime and activate tumor-specific Th1 $CD4^+$ T cells and cytotoxic $CD8^+$ T cells in the draining lymph node by presenting TAAs. Hundreds of TAAs have been described; they include neoantigens arising from somatic mutations, viral proteins, proteins overexpressed in specific tumors, embryonic and cancer testis antigens, as well as tissue-specific differentiation antigens [27]. Finally, effector T cells home to the tumor site, and, upon recognition of the specific antigen, kill their targets. However, this may not result in complete tumor eradication. Under subsequent selection pressure by lymphocytes and IFN-γ, surviving tumor cells will — owing to their genetic

instability and high mutation rate — evolve into more suppressive and less immunogenic variants eventually escaping the immune response [14]. This concept termed 'cancer immunoediting' was proposed by the Schreiber group, expanding upon the idea of cancer immunosurveillance, in light of the fact that individuals develop cancer despite a functional immune system.

1.2.2 Tumor immune escape

Tumors escape cytolytic attack of the immune system by employing several immune evasion and suppression mechanisms, which eventually results in a clinical manifestation of the malignancy.

Tumors can avoid T cell recognition by losing tumor antigens, downregulating MHC class I molecules or deregulating antigen-processing machinery [28]. Moreover, tumors can shed ligands for the activating receptor NKG2D on $CD8^+$ T cells and NK cells, which causes receptor degradation and impairment of immune responses [29, 30]. While acquiring resistance to cell death by upregulating anti-apoptotic proteins, tumors can exploit their expression of Fas ligand (FasL) to kill lymphocytes that express the death receptor Fas [31]. Many tumors acquire ligands for the receptor programmed death 1 (PD-1) expressed on activated T and NK cells, inducing immune cell anergy and dysfunction upon ligation. In addition, tumors can release a plethora of immunosuppressive, tumor-promoting, and angiogenic factors, such as ROS, transforming growth factor beta (TGF-β), IL-10, prostaglandin E2 (PGE2), CXCL8, and vascular endothelial growth factor (VEGF).

The chronic inflammatory milieu, created by tumors, promotes recruitment and induction of suppressive immune cell populations. Tregs, a subset of $CD4^+$ T cells characterized by high expression of the IL-2 receptor CD25 and transcription factor FoxP3, are crucial in the maintenance of self-tolerance. When recruited to, or induced in the TME, Tregs substantially suppress anti-tumor immune responses. Tregs not only deplete IL-2 required for lymphocyte proliferation and activation but also secrete TGF-β, IL-10, and IL-35 causing downregulation of activating receptors on T and NK cells, thereby inhibiting their effector functions [32]. Recently, the molecular mechanism of the inhibitory activity of TGF-β has been found to involve repression of the mammalian target of rapamycin (mTOR) pathway, resulting in disrupted NK cell metabolism, cytotoxicity, and expression of activating receptors [33, 34]. Further, Tregs have been shown to generate extracellular adenosine that can either directly suppress T and NK cell responses by acting on their adenosine A2A receptors, or indirectly through induction of tolerogenic DCs [35].

MDSCs, a heterogeneous population of immature cells of monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC) lineages arising in pathologic conditions, are another major immunosuppressive cell subset responsible for the impairment of NK and T cell-mediated anti-tumor responses. Induced by a number of inflammatory mediators in the TME, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, and PGE2 [36, 37], MDSCs employ a multitude of inhibitory mechanisms. Similar to Tregs, MDSCs can produce suppressive cytokines, such as IL-10 and TGF-β, but also PGE2 that can potently inhibit effector lymphocytes [38]. MDSCs also induce NK cell anergy through the inhibitory activity of membrane-bound (mb)TGF-β [39]. Moreover, MDSCs express high levels of arginase-1 and inducible nitric oxide synthase (iNOS) that catabolize L-arginine into urea and ornithine, and into NO and citrulline, respectively [40]. This depletes the essential amino acid required for T cell proliferation and expression of CD3ζ, the main signal-transduction domain of the TCR [40, 41]. MDSCs, as well as tumors, have also been shown to express the enzyme indoleamine 2,3-dioxygenase, thereby limiting access to tryptophan and generating the metabolite kynurenine, which is suppressive to both T and NK cells [42, 43]. ROS and reactive nitrogen species constitute yet another MDSC-derived suppressive mechanism inducing CD3ζ downregulation and T cell apoptosis [40].

A number of other immune and stromal cells, many of which are insufficiently studied, are recruited or subverted in the inflammatory and hypoxic TME to aid tumor progression and suppress anti-cancer immune responses. They include M2 TAMs that produce VEGF to promote angiogenesis, type II natural killer T cells that secrete immunosuppressive cytokines [44], type 2 neutrophils that generate factors boosting tumor angiogenesis and metastasization [45], regulatory B cells that can produce IL-10 and suppress T cell effector function [46], and cancer-associated fibroblasts that have been shown to downregulate activating receptors on NK cells and impair their cytotoxicity [47].

2 NATURAL KILLER CELLS

2.1 NK CELL SUBSETS

As part of the innate immune response, NK cells, constituting 5–15% of peripheral blood lymphocytes, play a crucial role in immunosurveillance through early elimination of virus-infected and tumor cells, and production of pro-inflammatory cytokines. NK cells were discovered in 1975 independently by Kiessling *et al.* [48] and Herberman *et al.* [49] due to their inherent or 'natural' ability to kill tumor cells without prior immune sensitization. Since then, great progress has shed light on NK cell development, phenotype and function, adding increasingly more pieces to a puzzle that is more complex than anticipated (Table 1).

Human circulating NK cells are traditionally divided based on their differential surface expression of CD56 (NCAM-1) into phenotypically and functionally distinct $CD56^{bright}$ and $CD56^{dim}$ subsets [50]. $CD56^{bright}$ NK cells are known to produce high levels of immunomodulatory chemokines and cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α, in response to DC-derived activating cytokines (combinations of type I IFNs, IL-2, IL-12, IL-15, and IL-18) [51]. The $CD56^{dim}$ subset, on the other hand, can effectively kill target cells through the release of cytotoxic granules containing perforin, granzymes, and other lytic proteins into an immunological synapse. Perforin permeabilizes the target cell, enabling entry of granzymes that initiate apoptosis [52]. In addition, NK cells can induce apoptosis through the ligation of tumor-expressed death receptors by TNF-related apoptosis inducing-ligand (TRAIL) and FasL. Furthermore, $CD56^{dim}$ NK cells express high levels of the low-affinity FcγR (CD16) enabling them to perform ADCC [53]. More recently it has been found that the $CD56^{dim}$ subset can also secrete large amounts of cytokines and chemokines following recognition of target cells [54]. Most studies agree that $CD56^{bright}$ NK cells mature into terminally differentiated $CD56^{dim}$ NK cells, acquiring the expression of CD16, killer cell Ig-like receptors (KIRs, described in chapter 2.2.1) and CD57 [55, 56]. NK cells can be distinguished from other group 1 ILCs based on the expression of perforin and the transcription factor Eomesodermin [3].

Recently, 'adaptive' or 'memory-like' subsets of NK cells have been identified that, in contrast to previous assumptions, are long-lived and able to mount stronger responses to a secondary stimulation — characteristics of immunological memory. In mice, adaptive NK cells have been found to expand upon murine cytomegalovirus (MCMV) infection and could be induced in murine livers upon exposure to chemical haptens and *in vitro* by stimulation with IL-12, IL-18, and IL-15 [57]. In humans, acute CMV infection or CMV reactivation has been shown to cause lasting expansions of NK cell subsets expressing activating NKG2C or CD2, and KIRs with the

potential to provide protective immunity [58-61]. Recent research suggests that CMV, combined with IL-12 and IL-18 signaling, activates a transcriptional program that epigenetically silences the transcription factor PLZF in conventional NK cells [62]. Downregulation of PLZF is a hallmark of adaptive NK cells and associated with stochastic allelic silencing of different signaling proteins, resulting in diverse NK cell subsets with a predominant expression of NKG2C and self-KIRs [63]. While adaptive NK cells don't respond to IL-12 or IL-18, they react stronger than conventional $CD56^{dim}$ NK cells to target cell engagement by cytokine production.

Table 1. Milestones in NK cell research.

1975	First description of NK cells	[48, 49]
1985	'Missing-self' recognition hypothesis	[64]
1995	Identification of KIRs on human NK cells	[65]
2000	Indirect evidence for protective role of NK cells in cancer	[66]
2002	NK cell-mediated graft-versus-leukemia immunity	[67]
2005	Adoptive transfer of haploidentical NK cells	[68]
2006	Concept of 'NK cell education' First observation of NK cell memory responses in mice	[69] [70]
2012	Induction of memory-like human NK cells	[71]
2018	Numerous ongoing clinical trials of adoptive NK cell infusions, in combination with other therapeutic modalities	[72]

2.2 REGULATION OF NK CELL RESPONSES

Given that NK cells are 'ready-to-go' cytotoxic cells, their activation needs to be tightly regulated. This is accomplished through the integration of a plethora of signals from inhibitory and activating receptors (Table 2), adhesion molecules and cytokines [73, 74].

2.2.1 Inhibitory signaling

The mechanism of target cell recognition by NK cells without previous encounter was proposed by Kärre *et al.* [64] as the 'missing self' hypothesis, suggesting that NK cells could detect and lyse cells that downregulate the expression self-MHC class I molecules — present on all healthy

cells — during a viral infection or malignant transformation. This implies that by eliminating cells trying to escape T cell responses, NK cells complement T cell-mediated immunosurveillance. In humans, MHC class I molecules are recognized by two major receptor families: KIRs that bind classical human leukocyte antigen (HLA)-A, B, and C molecules, and C-type lectin-like receptors NKG2 that form heterodimers with CD94 to bind the non-classical HLA-E molecule [65, 73]. Inhibitory KIRs and NKG2A contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domain and generate inhibitory signals. Activating KIRs and NKG2C, on the other hand, provide activating signals through DAP12 adapter proteins with immunoreceptor tyrosine-based activating motifs (ITAMs).

The expression of KIRs is stochastic and independent of MHC, which results in the generation of a diverse KIR repertoire with NK cells expressing few or none of many possible KIRs [75]. Therefore, to achieve self-tolerance, the strength of NK cell responses is modulated by the interaction of inhibitory KIRs and NKG2A with cognate ligands during NK cell development in a process termed 'education' or 'licensing' [69, 76]. Only NK cells that are inhibited by self-MHC will mature to become fully responsive and licensed to kill upon missing self-recognition. NK cells lacking inhibitory KIRs will stay hyporesponsive. More recently, it has emerged that NK cell activation thresholds are tuned on a continuum like a 'rheostat', and that this process occurs even in mature NK cells transferred into new MHC environments [77, 78].

2.2.2 Activating signaling

As negative signals from inhibitory receptors dominate over activating signals to maintain self-tolerance, the balance needs to be shifted for full NK cell activation via engagement of activating receptors that recognize their ligands on target cells [74]. In addition to CD16, some of the main activating receptors on NK cells are NKG2D, DNAX accessory molecule (DNAM)-1, and natural cytotoxicity receptors (NCRs). With the exception of CD16, however, none of the activating receptors can induce cytotoxicity and cytokine production by resting NK cells alone, but they require the synergistic action of other receptors [79].

NKG2D is a C-type lectin receptor that is expressed on all human NK cells and $CD8^+$ T cells. NKG2D binds its ligands MHC class I-related chain (MIC)A and MICB as well as UL16-binding proteins (ULBP)1-6 as a homodimer, and signals through DAP10, inducing NK cell cytotoxicity, cytokine production, and survival pathways [73, 80]. The importance of NKG2D in NK cell mediated anti-tumor responses has been shown in a mouse model where NK cells rejected tumors with ectopic NKG2D ligand expression, overcoming MHC class I inhibition [81]. NKG2D ligands, while absent from healthy tissues, are induced upon cellular stress, such as DNA damage [82], and have been found to be expressed on many tumors, including CRC,

renal cell carcinoma (RCC), breast cancer, and acute myeloid leukemia (AML) [80]. However, soluble NKG2D ligands as well as chronic exposure to NKG2D ligands can abrogate NK cell function [30, 83].

Table 2. NK cell receptors and ligands.

Receptors	Ligands
Inhibitory	
KIR2DL, KIR3DL	HLA class I
NKG2A	HLA-E
ILT2 (LIR-1)	HLA class I, HLA-G, UL-18 (HCMV protein)
TIGIT	PVR, Nectin-2
Tactile (CD96)	PVR
Activating	
KIR2DS, KIR3DS	HLA class I
NKG2C	HLA-E
CD16	IgG
DNAM-1	PVR, Nectin-2
NKG2D	MICA, MICB, ULBP1-6
NKp30	B7-H6, BAG6, heparin or heparan sulfate
NKp44	Hemagglutinin, heparin or heparan sulfate
NKp46	Hemagglutinin, heparin or heparan sulfate
2B4	CD48
CD2	CD58 (LFA-3)

DNAM-1 (CD226) is an Ig family glycoprotein expressed on the majority of human NK cells, T cells and monocytes. It is imperative for tumor immunosurveillance as it mediates intracellular adhesion, IFN-γ production, and cytotoxicity of T and NK cells against a wide range of tumor cells through the interaction with its cellular stress-induced ligands PVR (CD155) and Nectin-2 (CD112) [84-89]. DNAM-1 forms a functional pair with the integrin LFA-1 in the immune synapse, with the latter mediating granule polarization, and enabling DNAM-1 signaling and efficient target cell killing [90, 91]. Importantly, DNAM-1 shares its ligands with two other receptors, TIGIT and CD96, both of which counteract DNAM-1 functions on NK cells [92, 93].

NCRs, which include NKp46, NKp30 and NKp44, are transmembrane proteins of the Ig family. They bear ITAMs in their cytoplasmic tail and signal though associated FcεRγ and/or CD3ζ dimers or through the adapter molecule DAP12 to mediate cytotoxicity against transformed cells [94]. While NKp46 and NKp30 are constitutively expressed on NK cells, NKp44 is induced upon activation. What NCRs recognize on their target cells remains mostly elusive, but viral hemagglutinins as well as heparin and heparan sulfates, which are upregulated on cancer cells,

have been identified as ligands [95, 96]. The transmembrane protein B7-H6, which binds NKp30, is the most studied ligand in tumor immunosurveillance as its expression is confined to tumor cells [97].

2.3 REGULATION OF NK CELL MIGRATION

NK cells are present in different tissues, contributing to immunosurveillance, and can be mobilized to sites of inflammation and tumor growth in response to chemotactic cytokines, or chemokines for short. Chemokines regulate leukocyte trafficking through G protein-coupled receptors, the activation of which triggers cell polarization, migration, and adhesion. Competing or synergistic chemotactic gradients, receptor heterodimerization, and receptor cross-regulation complicate the interpretation of this highly complex process, particularly *in vivo* [98]. In addition, chemokine functions go beyond chemoattraction and include, for instance, regulation of NK cell cytotoxicity [99].

$CD56^{bright}$ NK cells in peripheral blood express CCR7, allowing them to enter SLTs in response to CCL19 and CCL21. In addition, they display high levels of CCR5, CXCR3 and CXCR4 (Figure 1) [100]. CXCR4, which binds CXCL12, provides the signal for NK cell retention in the bone marrow. The reduction of CXCR4-mediated retention during NK cell maturation, accompanied by the engagement of the sphingosine 1-phosphate (S1P) receptor-5, allows mature $CD56^{dim}$ NK cells to egress from the bone marrow during steady state [101]. Moreover, $CD56^{dim}$ NK cells express high levels of CXCR1, CXCR2, CX3CR1, and CXCR3, albeit the latter at lower levels than the $CD56^{bright}$ subset (Figure 1) [100, 102].

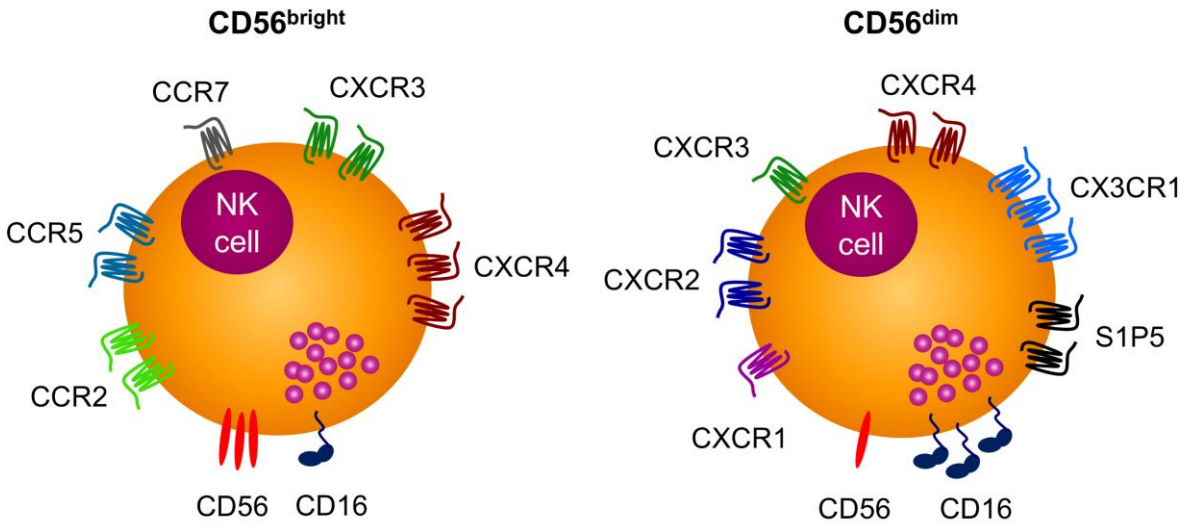

Figure 1. Chemokine receptors expressed on $CD56^{bright}$ and $CD56^{dim}$ NK cells.

NK cells have been shown to migrate to sites of acute inflammation via the chemokine receptors CCR2, CCR5, and CXCR3 [98, 101]. Infiltrating NK cells initiate a feed-forward mechanism promoting further chemotaxis of innate immune cells and DCs through the secretion of IFN-γ that induces the production of the CXCR3 ligands CXCL9, CXCL10, and CXCL11. The presence of specific chemokines in the TME, including CXCR3 ligands, CX3CR1 ligand CX3CL1, as well as CCL3 and CCL5 that bind CCR5, has been shown to promote trafficking of NK cells, as well as T cells, in murine tumor models [103, 104]. Moreover, intratumoral Th1 chemokines and infiltration of effector lymphocytes have been correlated with improved patient outcomes in a variety of cancers types, such as melanoma, RCC, CRC, and breast cancer [105-108]. However, a big fraction of tumors contains low levels of pro-inflammatory chemokines due to exclusion of immune cells or immunosuppression. On the other hand, ligands for CXCR2 are abundantly secreted by a variety of solid tumors, including melanoma, prostate cancer, and RCC [109, 110], in response to pro-inflammatory mediators, such as IL-1β and TNF-α [111]. However, NK cell migration toward CXCR2 ligands, which include CXCL1–3 and CXCL5–8, has so far only been studied *in vitro* [100].

2.4 NK CELLS IN TISSUES

Recent attention has turned to studying NK cells populating different tissues, such as the liver, uterus, salivary gland, and adipose tissues, where they can be found at varying frequencies [112, 113]. Constituting on average less than 10% of the NK cells in the peripheral blood, the $CD56^{bright}$ subset is enriched in SLTs and, among others, in the uterus, liver, stomach, and colorectal tissues [112, 113]. Tissue-resident $CD56^{bright}$ NK cells can be distinguished from peripheral blood NK cells through the presence of tissue retention mechanisms, such as a specific chemokine receptor repertoire, adhesion molecules, and CD69, an early activation marker that has been found to inhibit lymphocyte egress by suppressing S1P receptor-1 function [113, 114]. $CD56^{bright}$ NK cells populating the lymph nodes, spleen, bone marrow, and liver have been found to express CXCR6 and CD69 [115-117]. Uterine NK cells, on the other hand, don't display chemokine receptors but express the CD49a and CD103 subunits of integrin receptors that bind extracellular matrix proteins and E-cadherin, respectively [118]. In addition, uterine and liver-resident NK cells have been reported to have reduced expression of DNAM-1 [113, 117]. Tissue-resident NK cells are poor cytotoxic mediators due to low levels of perforin and lack of CD16 expression [118]. However, they produce cytokines, such as IFN-γ, albeit at lower levels compared with their circulating counterparts [113]. Instead their function appears to be priming of Th1 responses in the SLT, vascular remodeling associated

with pregnancy in the decidua, and non-cytotoxic immunosurveillance in the liver and other tissues in response to cytokine stimulation [113, 118].

2.5 NK CELLS IN CANCER

2.5.1 NK cells mediate anti-tumor immunity

Several lines of evidence support the importance of NK cells in anti-tumor immunity. Depletion experiments of NK cells with anti-asialoGM-1 or anti-NK1.1 antibodies in allograft mouse models of cancer, such as lymphoma, melanoma, lung, and prostate cancer, have established a crucial role for NK cells in controlling tumor growth and metastatic spread [119, 120]. At the same time, NK cell infusion into NK cell-depleted mice has been shown to reinstate resistance to metastasization [119]. A recently uncovered mechanism suggests that remodeling of the extracellular matrix in tumors by NKp46-induced IFN-γ leads to reduced metastasis formation [121]. Furthermore, a study using mouse models of genetic NK cell deficiency has found that NK cells reduce the incidence of chemically-induced sarcoma in the absence of adaptive immunity through the activation of M1 macrophages [122].

Human autologous or allogeneic NK cell have been shown to recognize and kill primary tumor cells from patient biopsies *in vitro*. Lysis of hematologic cancer cells, obtained from AML, acute lymphoid leukemia (ALL), and multiple myeloma (MM) patients, has been found to be primarily enabled via ligation of NKG2D or DNAM-1, and facilitated by low HLA class I expression and a KIR ligand mismatch [123]. Cells isolated from advanced solid tumors have also been observed to be susceptible to lysis by activated NK cells. Neuroblastoma cells have been found to have negligible HLA class I expression, and to be killed by allogeneic NK cells, albeit at varying degrees, which could be explained by differential expression of PVR and engagement of DNAM-1 [86]. Likewise, killing of ovarian cancer cells by allogeneic NK cells has been shown to inversely correlate with tumor HLA class I expression, and to be mediated primarily by the DNAM-1/PVR axis, with a complementary role for NKG2D engagement [85]. Moreover, *ex vivo* expanded allogeneic NK cells have been shown to kill tumor cells isolated from RCC, CRC, ovarian, and gastric cancer patients in a KIR-ligand mismatch setting, whereas KIR-ligand matched tumors were resistant to lysis [124]. Worthy of note, both allogeneic and autologous, activated NK cells have recently been reported to preferentially target cancer stem cell (CSC)-like populations in fresh patient-derived tumor samples. NK cell-mediated lysis of CSC-like cells from sarcoma and pancreatic cancer patients was shown to be dependent on their overexpression of NKG2D ligands, while CSC-like cells in CRC were targeted via overexpressed ligands for NKp30 and NKp44 [125, 126].

A prospective study in more than 3,600 individuals with an 11-year follow-up has correlated high and medium cytotoxic activity of peripheral blood NK cells with a reduced risk to develop cancer, providing indirect evidence for a role of NK cells in tumor immunosurveillance [66]. Assessing IFN-γ production by NK cells, a recent prospective study in more than 800 individuals at high risk of CRC came to a similar conclusion [127]. High intratumoral NK cell levels have been correlated with an improved survival in many types of cancer. Indeed, intratumoral infiltration of NK cells has been associated with a good prognosis in CRC, RCC, gastric, esophageal cancer, and squamous cell lung carcinoma [128-132]. The use of CD57 as phenotypic marker for NK cells in these studies, however, is not ideal, for CD57 is also expressed on a subset of T cells. Studies that have evaluated intratumoral NK cell infiltration using staining for CD56 in NSCLC and androgen-deprived prostate cancer patients associated it with better patient outcomes [133-135]. However, CD56 alone is not a suitable immunohistochemistry marker either, for it can be found on a fraction of T cells and various other tissues. A study that detected NK cells based on the expression of the NK cell-specific marker NKp46 found no correlation of intratumoral NK cell density and clinical outcome in early stage NSCLC [136]. In contrast, NK cell density assessed by NKp46 in gastrointestinal tumors appeared to be inversely correlated with the number of metastases [137]. However, despite the shortcomings of some studies, collectively, they indicate a beneficial role for NK cells in cancer prognosis.

2.5.2 NK cells are negatively regulated in cancer

While tumors can induce NK cell activation, NK cells found in cancer patients are generally negatively affected. In hematological malignancies, such as AML, NK cells are profoundly inhibited and have reduced expression of NCRs, which results in an impaired ability to perform cytotoxicity and produce cytokines [138, 139]. While circulating NK cells in patients with solid tumors can also be suppressed, the effects are much more pronounced on tumor-infiltrating NK cells.

A large variety of solid tumors, including lung and breast cancer but not kidney cancer, are highly enriched in $CD56^{bright}CD16^{low}$ NK cells, which appears to be reflective of the chemokine milieu in the TME [112, 140]. Tumor-infiltrating NK cells have been shown to have suppressed phenotypes characterized by downregulated expression of activating receptors, such as DNAM-1, NKG2D, NCRs, and CD16, and increased expression of inhibitory receptors, such as NKG2A and ILT2, when compared to NK cells in the peripheral blood or healthy tissues in lung, breast, prostate, and kidney cancer patients [136, 141-143, **paper III**]. Moreover, PD-1 has recently been reported to be induced on tumor-associated NK cells of patients with ovarian

and hepatocellular carcinomas [144, 145]. Conversely, some studies have also observed an upregulation of activation markers, including CD69 and NKp44, on intratumoral NK cells [141, 142].

The suppressed phenotype of intratumoral NK cells is accompanied by impaired natural cytotoxicity, ADCC, and cytokine production [136, 137, 141-143, 146]. In ovarian cancer patients, tumor-associated NK cells have been shown to fail to target autologous tumors via ADCC, or degranulate against K562 target cells due to reduced CD16 and DNAM-1 expression, respectively [146]. In addition, PD-1$^+$ NK cells in ovarian cancer patients have been observed to degranulate less than their PD-1$^-$ counterparts [144]. Moreover, lysis by intratumoral NK cells from RCC patients has been found to be inhibited via the NKG2A/HLA-E axis [143]. In addition, HLA-G expressed in over 50% of clear cell (cc)RCCs [147] has been shown to impair NK cell synapse formation via ligation of ILT2, thereby inhibiting cytotoxicity and cytokine production [148]. In patients with breast and prostate cancer, suppression of NK cell functionality has been associated with the presence of TGF-β and PGE2 in the TME and could be partially relieved upon TFG-β neutralization [141, 142]. In addition, mbTGF-β on Tregs of patients with gastrointestinal tumors has been shown to inhibit NK cell cytotoxicity, IFN-γ production, and NKG2D expression [149].

3 IMMUNOTHERAPY OF CANCER

Although the foundations of cancer immunotherapy were laid as early as the 1890s with 'Coley's toxins', heat-inactivated bacteria mixtures, that could cure a number of patients with advanced cancer [150], it remained an obscurity few believed in for nearly a century. Today, cancer immunotherapy is a rapidly advancing field that has generated impressive breakthroughs in recent years. The purpose of cancer immunotherapy is to target key limiting steps in the cancer-immunity cycle in order to generate or augment effective anti-tumor immune responses.

There are currently more than 2,000 immuno-oncology agents in preclinical and clinical development [151]. The largest category are cancer vaccines, with DC-mediated priming of TAA-specific T cell responses lying at the heart of this approach. Vaccine strategies include injection of TAAs as synthetic peptides or encoded in DNA vectors combined with an adjuvant or, alternatively, *in vitro* maturation of peripheral blood-derived monocytes into DCs that are then loaded with TAAs in the form of peptides, proteins, nucleic acids, or lysates from autologous or allogeneic tumors and infused into the patient [152]. Despite some successes, clinical benefit has been generally minimal. Sipuleucel-T, a DC-based vaccine, approved in 2010 for patients with castration-refractory prostate cancer prolonged overall survival by only 4.1 months [153], but its FDA approval paved the way for future cell-based therapies. With better understanding of the immunization process and technological advances, cancer vaccines will, hopefully, become clinically more effective. Other immunotherapeutics include immunomodulatory cytokines and other agents, oncolytic viruses and bispecific antibodies; however, the two most outstanding approaches are immunomodulatory antibodies, in particular T cell 'checkpoint inhibitors', as well as adoptive cell therapies (ACTs).

3.1 IMMUNOMODULATORY ANTIBODIES

Immune effector cells that recognize tumors may be held back by molecular pathways that suppress their activation and functions. Immunomodulatory antibodies work by either blocking key inhibitory receptors on activated T cells, as well as NK cells, or their respective ligands, or by activating co-stimulatory receptors on effector cells, thereby unleashing anti-tumor immune responses. Targeting of the inhibitory 'checkpoints' cytotoxic T lymphocyte antigen 4 (CTLA-4) and PD-1 on T cells has proved clinically effective across a multitude of tumor types and transformed cancer immunotherapy.

CTLA-4 plays a central role in the maintenance of immunological tolerance by dampening T cell responses in secondary lymphoid organs. The inhibitory receptor is expressed on activated

T cells — predominantly $CD4^+$ T cells — and competes with the co-stimulatory receptor CD28 for their shared ligands CD80 and CD86 on the surface of APCs [154]. The finding that CTLA-4 blockade mediates tumor rejection and regression in murine models [155] led to clinical testing and, in 2011, FDA approval of the anti-human CTLA-4 blocking antibody ipilimumab for the treatment of patients with advanced melanoma [156]. A meta-analysis of over 1,800 individuals revealed that ipilimumab conferred durable survival to 21% of the patients with advanced melanoma, rendering it a chronic disease [157]. In other solid tumors, including kidney, lung, and prostate cancer, ipilimumab monotherapy has shown only modest clinical benefit, and is now evaluated in combination with other treatments. In addition to disabling negative signaling in T cells, anti-CTLA-4 has been demonstrated to deplete tumor-infiltrating Tregs by ADCC in murine models [158], thus potentially affecting T and NK cell anti-tumor responses in the TME.

PD-1 expression is also induced on T and NK cells upon activation, albeit at a delayed time point. Persistent PD-1 signaling leads to T and NK cell exhaustion and dysfunction as well as induction of apoptosis through ligation of PD-L1 and PD-L2 [159-161]. PD-L1 is upregulated in many solid tumors, both on tumor cells and immune cells, whereas PD-L2 expression is mostly restricted to activated DCs. The success of ipilimumab has accelerated the clinical development and FDA approval of antibodies blocking the PD-1/PD-L1 pathway. Generating durable survival benefits in advanced melanoma patients, the first PD-1 blocking antibodies to be approved were pembrolizumab and nivolumab in 2014 [162, 163]. Since then, PD-1 blockade has demonstrated clinical efficacy across a broad range of solid tumors and hematological malignancies [164]. A surge of approvals followed in recent years, bringing anti-PD-1 antibodies into earlier treatment phases and broadening the indications to include NSCLC, Hodgkin's lymphoma, RCC, and head and neck cancer, to name a few. With the approval of atezolizumab for the treatment of urothelial carcinoma in 2016, the first PD-L1-blocking antibody entered the stage. There are currently five approved agents targeting the PD-1/PD-L1 pathway, with additional 159 in preclinical and clinical development [151]. While anti-PD-1 monotherapy is more effective than anti-CTLA-4 monotherapy, combination therapy with the two agents — albeit associated with more severe immune-related toxicities — is clinically even more beneficial, improving response rates and overall survival rates in advanced melanoma and metastatic CRC with DNA mismatch repair-deficiency [165, 166].

3.2 ADOPTIVE CELL THERAPY

For adoptive cell therapy, immune cells are isolated from the patient or a donor, activated and expanded *ex vivo* under optimal conditions and in absence of suppressive factors, and

(re-)infused. The first human trials were pioneered by Rosenberg *et al.* who used lymphokine-activated killer (LAK) cells, which are IL-2 activated autologous peripheral blood mononuclear cells (PBMCs), with high-dose IL-2 to treat immune-sensitive tumors, including melanoma and RCC [167]. The observed cytotoxicity of LAK cells against tumors was found to be primarily mediated by NK cells. However, the clinical benefit of this approach was limited, while IL-2 induced toxicities, including capillary leak syndrome, could be severe [168]. When it was found that host lymphocytes compete with the infused cells for cytokines and space, pre-conditioning regimens with lymphodepleting chemotherapy and/or total body irradiation were included into ACT treatment protocols, greatly improving patient outcomes [169], not least due to the depletion of Tregs.

A major advancement for ACT in the 1980s was the use of tumor-infiltrating lymphocytes (TILs) grown out from excised tumors in high-dose IL-2 *in vitro* cultures, which due to their high degree of tumor-reactivity were shown to be effective in patients with advanced melanoma [170]. A more recent report on 93 patients with metastatic melanoma treated with TIL therapy, demonstrated up to 72% objective response rate and a 36% 3-year survival, with potential cures among the complete responders [171]. However, TIL therapy has mostly been limited to melanoma, possibly due to relatively easy access to tumor tissue and high mutation frequency providing a large amount of unique TAAs.

Peripheral blood T cells genetically engineered to recognize TAAs offer a solution when TILs can't be generated. One approach relies on the introduction of cloned TCRs with strong affinity for a TAAs presented in a MHC context. In a recent trial, T cells transduced with TCRs specific for cancer testis antigen NY-ESO-1 achieved tumor regression in over 50% of patients with metastatic melanoma and sarcoma [172]. A drawback of this approach, however, is that tumors may downregulate MHC class I expression or antigen presentation to escape recognition.

Alternatively, T cells can be redirected by the introduction of a chimeric antigen receptor (CAR) that consists of a single chain variable fragment (scFv) with specificity for a TAA, linked to intracellular signaling domains. In contrast to TCRs, CARs recognize antigens independent of MHC presentation, but they are limited to surface-expressed antigens. Since the initial design, modifications of the intracellular CD3ζ domain by addition of co-stimulatory domains have improved the activity, *in vivo* persistence, and efficacy of CAR T cells. A multitude of clinical trials using CAR T cells targeting CD19 in B cell malignancies have shown remarkable and often durable responses. According to an updated report, CD19-CAR T cells achieved complete remissions in 90% of children and adult patients with ALL [173]. Indeed, these clinical successes have resulted in FDA approvals for two CD19-targeting T cell products in

2017. However, CAR T cells struggle to achieve clinical efficacy in solid tumors, not least due to inefficient homing and suppression in the TME. Treatment of metastatic RCC patients with CAR T cells directed against carbonic anhydrase IX, an antigen expressed on almost all clear cell (cc)RCCs, showed no clinical benefit [174]. Instead it highlighted another main challenge for engineered T cell therapies — 'on-target off-tumor' toxicity due to non-exclusive tumor expression of antigens. However, strategies are being developed to address issues of safety, tissue selectivity, persistence, trafficking, and immunosuppression, and although the majority of clinical trials are conducted in hematological malignancies, ACT approaches for solid tumors are picking up pace [175].

3.3 NK CELL THERAPY

Given that NK cells don't rely on immune priming and tumor antigens and are easily accessible from peripheral blood, they represent an attractive approach for immunotherapy.

In a seminal study, Ruggeri *et al.* demonstrated a potent clinical effect of alloreactive NK cells in AML patients in the setting of T cell-depleted hematopoietic stem cell transplantation (HSCT) [67]. HLA-mismatched, alloreactive donor NK cells mediated a potent graft-versus-leukemia effect, increasing the 5-year event-free survival from 5% to 60% and reducing the relapse probability from 75% to 0% compared with HLA-matched NK cells. While HSCTs permanently introduce a competent immune system, they are associated with high risks and toxicities. Miller *et al.* have demonstrated in patients with advanced cancers that haploidentical adoptive transfer of expanded NK cells together with subcutaneous IL-2 is a safer treatment [68]. In addition, the NK cell transfer led to remission in 26% of AML patients. Numerous clinical trials, primarily in hematological malignancies but also in neuroblastoma, have since then tested and continue to test this approach [72]. However, the transfer of expanded autologous NK cells in patients with metastatic melanoma, RCC, and advanced gastrointestinal tract cancers hasn't resulted in clinical responses [176, 177]. These and other studies have made clear that (a) NK cell persistence *in vivo* is crucial for clinical efficacy, (b) while IL-2 can be administered safely, it expands Tregs, (c) autologous NK cells are inhibited by self-HLA molecules, and (d) solid tumors are more difficult to target than hematological cancers.

3.3.1 NK cell sources and *ex vivo* expansions

NK cell-based therapies require large numbers of cells that need to be enriched from peripheral blood and expanded *ex vivo*. When employing allogeneic NK cells, it is crucial to deplete T and B cells using immunomagnetic beads not only to achieve higher NK cell purity but also to reduce graft-versus-host disease and the risk for antibody responses to host erythrocytes and

autoimmunity [178]. For autologous NK cells, expansion protocols without NK cell separation steps have been developed [177, 179]. In addition to peripheral blood, NK cells can be generated from HSCs in umbilical cord blood [180, 181], or from embryonic or induced pluripotent stem cells that are first differentiated into HSCs [182], making them a potential 'off-the-shelf' cell therapy. NK cell lines purified from malignant NK cell clones, particularly NK-92 cells, are gaining more attention as 'off-the-shelf' immunotherapeutics due to easier logistics in terms of culture and genetic modification potential inherent to cell lines as opposed to primary cells. Indeed, despite the lack of CD16 expression and IL-2 addiction, both of which can be genetically adjusted, NK-92 cells engineered to express CARs are on the rise [183].

Several *ex vivo* NK cell expansion protocols have been developed based on stimulation with cytokine cocktails or irradiated feeder cells in combination with cytokines, which, in addition to upregulating NK cell activation markers and enhancing their effector functions, can increase NK cell numbers 100- to over 30,000-fold in 2–3 weeks. Clinically applied feeder cell-based strategies include irradiated PBMCs and cell lines. K562 cells converted into artificial APCs (aAPCs) by introducing the expression of mbIL-15 and the ligand for the co-stimulatory receptor 4-1BB (CD137) were shown to yield 1,000-fold expansions [184]. They were, however, limited by NK cell exhaustion and replicative senescence, which could be overcome by K562 aAPCs expressing mbIL-21, resulting in increased telomere length and catapulting NK cell expansion potential beyond 30,000-fold [185]. Another protocol utilizes Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) and high-dose IL-2 [186, 187]. It has recently been modified by adding IL-21 on the first day of the expansion, which resulted in sustained NK cell proliferation and high cytokine production [188]. Furthermore, it could be beneficial to infuse specific NK cell subsets, skewed toward matured or memory-like phenotypes. Short NK cell activation with IL-12, IL-15, and IL-18 has been found to induce a memory-like NK cell phenotype with enhanced IFN-γ secretion and cytotoxicity upon re-stimulation [71]. While the efficacy of this cell product is currently being evaluated in the clinic for the first time, this cytokine cocktail has recently been applied to NK cells expanded with K562-mbIL-21 feeder cells to yield high numbers of these highly potent cells [189]. Another recently developed approach is the induction of mature $CD57^+$ NK cells with improved effector functions by culture with IL-15 and an inhibitor of the GSK3 kinase [190].

3.3.2 Improving NK cell therapy of solid tumors

While NK cell therapies have demonstrated clinical benefits in hematological malignancies, they are facing ample challenges to succeed in solid tumors.

3.3.2.1 Enhancing in vivo proliferation and persistence

A barrier that infused NK cells targeting both hematological and solid cancers need to overcome is long enough persistence in the patient to exert a meaningful anti-tumor response. NK cells rely on cytokines for the stimulation of their proliferation, cytokine production and cytotoxic function. The most studied and clinically advanced cytokines are IL-2 and IL-15. High dose recombinant human (rh)IL-2 is used in the clinic, particularly in patients with advanced RCC and melanoma, with modest response rates and serious drawbacks, including severe toxicities and expansion of Tregs [168]. Due to its different biology that doesn't stimulate Tregs, rhIL-15 is now being clinically investigated in advanced solid tumors with first results showing that daily administration of rhIL-15 induces proliferation of circulating NK cells, however without objective clinical responses [191-193]. Since IL-15 requires trans-presentation in a complex with IL-15Rα to be fully effective, a fusion protein has been developed that consists of a mutated high-affinity IL-15 (superagonist), linked to the IL-15Rα sushi domain, fused to an Fc domain (ALT-803) [194]. After having demonstrated improved NK cell function and anti-tumor responses in mouse models [194], ALT-803 has entered clinical trials for solid cancers and relapsed hematological malignancies. The first results in patients with hematological malignancies relapsed after HSCT have shown a NK cell expansion and stimulation activity of ALT-803 similar to that of rhIL-15, but associated with less toxicities, resulting in clinical benefit in 19% of the patients [195].

3.3.2.2 Improving homing to tumor sites

NK cells activated and expanded *ex vivo* have an altered chemokine receptor repertoire and may lack homing cues to tumor sites [196]. At the same time, tumors change the chemokine composition in their microenvironment to promote neoplastic growth, angiogenesis and attract immunosuppressive cells [197]. It is not known how many of the adoptively transferred cells make it to the patient tumors — according to calculations from one study, it may be as little as 0.01–0.04% [198] — however, trafficking to the tumor site has been associated with improved clinical responses and is now being increasingly acknowledged as a potential limitation of ACTs [199].

One approach to enhance NK cell infiltration into tumors is to induce or increase Th1-type chemokines in the TME. Mouse models have shown that local IFN-γ or CXCL10 injections induce intratumoral infiltration of $CXCR3^+$ NK cells, thus improving survival [104]. We have explored this approach in a preclinical model in the setting of ACT using human expanded NK cells (**paper I**). Other mouse studies have shown that the secretion of CXCR3 ligands by DCs and myeloid cells can be induced by intratumoral injection of Sendai virus particles and PD-1

blockade in combination with ACT, respectively, promoting intratumoral infiltration of lymphocytes [200, 201]. In addition, localized radiation treatment of tumors has been shown to induce IFN-γ-mediated production of CXCR3 ligands [202]. Furthermore, it has recently been found in preclinical models that epigenetic histone and DNA methylation can silence the secretion of CXCL9 and CXCL10 by the tumor [203, 204]. Treatment with epigenetic modulators inhibiting histone or methyl transferase activity has been shown to induce the production of Th1-type chemokines, increase NK and effector T cell infiltration into the tumor, and enhance the therapeutic effect of PD-1 or CTLA-4 blockade in murine ovarian cancer models [204, 205].

Another approach is to exploit the chemokines present at the targeted site and modify the chemokine receptor expression on NK cells accordingly (**paper II**). This concept was first explored by the Hwu group that genetically modified human T cells to express CXCR2, thereby improving their migration toward melanoma supernatants [206]. Subsequently, they transduced murine antigen-specific T cells with CXCR2 and demonstrated their increased accumulation at the tumor site, which, in combination with DC vaccination and IL-2, mediated superior anti-tumor responses compared with control T cells [207]. Furthermore, murine and human CAR T cells have been engineered to express CCR2 and CCR4, resulting in increased infiltration and anti-tumor responses in xenograft mouse models [208, 209]. CXCR2-transduced T cells in combination with high-dose IL-2 are now being tested in a clinical trial in metastatic melanoma patients. With regards to NK cells, transient expression of CCR7 on expanded human NK cells has been achieved by a process of membrane transfer from feeder cells called trogocytosis, enabling migration to lymph nodes in an immunocompromised mouse model [210]. Moreover, a human NK cell line has been transduced with CXCR4, resulting in enhanced NK cell infiltration and improved survival in a glioblastoma xenograft model [211].

3.3.2.3 Reducing suppression in the tumor microenvironment

One of the major hurdles for NK cells in solid tumors is the suppression by soluble factors and inhibitory cells in the TME. With TGF-β being a major suppressor of NK cells, several antibodies and small molecule inhibitors blocking its signaling have been developed. Galunisertib, a small molecule inhibitor interfering with the signaling cascade downstream of TGF-β receptors, has shown encouraging results in preclinical models and early clinical trials and is now being further clinically evaluated [212]. An alternative approach is to neutralize TGF-β in the TME. Recently, a fusion protein containing the TGF-β receptor II that functions as a molecular 'trap' fused to an antibody against PD-L1 has been developed [213]. The fusion

protein has demonstrated promising anti-tumor activity in mouse models and is now being tested in clinical trials.

Moreover, it is necessary to eliminate suppressive cells, including Tregs and MDSCs. Expansion of Tregs in the setting of ACT can be avoided by replacing IL-2 with alternative cytokines, as described earlier, or by using IL-2 fused to diphtheria toxin, which has been shown to selectively deplete Tregs and improve remission rates and disease-free survival in refractory AML patients receiving NK cell infusions [214]. Furthermore, it has been shown *in vitro* and in murine tumor models that Tregs can be selectively depleted through anti-CTLA-4-enabled ADCC by NK cells and macrophages [23, 158].

It is challenging to target MDSCs in human cancer. One of the clinically most advanced approaches is to reduce chronic inflammation that drives the development of MDSCs through the activity of PGE2. Pharmacologic inhibition of cyclooxygenase-2 (COX-2), a key enzyme in the synthesis of PGE2, has been shown to reduce MDSC accumulation and tumor growth in tumor-bearing mice [215]. The use of COX-2 inhibitors has demonstrated a preventive effect on cancer incidence in a large number of patients [216]. In addition, standard therapeutic regimens, such as the chemotherapeutic agent doxorubicin and an inhibitor of VEGF, have been shown to selectively deplete MDSCs from the TME in a preclinical model of breast cancer and in patients with advanced RCC, respectively [217, 218].

3.3.2.4 Overcoming inhibition

During the process of immunoediting, tumors can upregulate HLA class I expression to evade NK cell responses. Blocking antibodies have been developed to release the inhibition through inhibitory KIR and NKG2A. Although initial clinical trials of an anti-KIR antibody, IPH2101, in AML and MM patients looked promising [219, 220], a phase II trial failed to show clinical benefit in patients with smoldering MM [221]. Subsequently, it was shown that IPH2101 mediated trogocytosis of KIR2D receptors on NK cells, preventing them from becoming 'educated' and fully functional [222]. Clinical trials are now evaluating the safety and efficacy of other anti-KIR antibodies as well as of an anti-NKG2A antibody [223]. Naturally, it should be important to explore their effect on NK cell education, too.

NK cells also express other inhibitory immune checkpoints, some of which are induced upon prolonged activation to limit tissue damage from an overactive immune response. In a cancer patient, however, they restrict anti-tumor immunity. PD-1 blockade on T cells has led to unprecedented clinical benefit in many cancer patients. Although PD-1 expression on NK cells is low even upon induction, it is likely that anti-PD-1 treatment may be beneficial. In nude

mice, PD-1 blockade was shown to reduce tumor growth, which was dependent on NK cell activity [145]. The effects of PD-1 blockade on NK cells from human trials have so far not been reported. Despite being frequently cited as a PD-1-targeting antibody with NK cell modulatory activity, pidilizumab (formerly CT-011), which has shown clinical efficacy in recent phase II trials, has turned out to primarily bind Delta-like 1 [224]. Other NK cell checkpoints that may be of interest to target on NK cells are TIGIT, Tim-3, and Lag-3, for which the first blocking antibodies are currently in early clinical trials. However, with the exception of TIGIT, which has been shown to suppress cytotoxicity [92, 225], the roles of these checkpoints in NK cells are not well understood yet. Nevertheless, both the inhibition of TIGIT and of Lag-3 has been shown to enhance NK cell function *in vitro* and anti-tumor activity in a preclinical model, respectively [225, 226].

Another way to bypass inhibition and activate NK cell cytotoxicity is to elicit strong activating signaling through CD16. Many commercially and clinically successful antibodies, including rituximab that targets CD20, trastuzumab that binds HER2/neu, and cetuximab that recognizes the epidermal growth factor receptor (EGFR), rely for their therapeutic effect, in part, on NK cell-mediated ADCC. Novel bispecific and trispecific killer cell engagers (BiKEs and TriKEs) consisting a scFv from an antibody binding CD16 linked to one or two scFvs specific for tumor antigens, have been developed to establish an immunological synapse and trigger tumor antigen-specific ADCC by NK cells [223]. Instead of a scFv, a TriKe can contain a cytokine, such as IL-15, to activate NK cells *in vivo*. BiKEs and TriKEs with an array of tumor specificities have been designed. The first TriKE, targeting CD33 and containing IL-15, has just entered a clinical trial for patients with refractory AML and high risk myeloid dysplastic syndrome (MDS) [223]. BiKEs and TriKEs follow the footsteps of bispecific antibodies triggering T cell activation through an anti-CD3 domain. Currently, catumaxomab directing T cells to EpCAM-expressing tumor cells in malignant ascites and blinatumomab targeting CD19 in B cell ALL are approved for therapeutic use [227]. In addition to promoting tumor cell lysis by T cells, catumaxomab can bind with its Fc region FcγR-expressing cells, such as NK cells and macrophages, and induce ADCC and phagocytosis.

Alternatively, NK cells can be equipped with a CAR, conferring tumor specificity, an activation signal via CD3ζ and co-stimulatory signaling domains, and, in newer CAR generations, a proliferation signal [228]. Similar to CAR T cells, preclinical and clinical CAR NK cell development mainly focuses on targeting CD19 in B cell malignancies [228, 229]. Most recently, CD19-targeting CAR NK cells derived from umbilical cord blood that contain IL-15 and an inducible suicide gene based on caspase-9 have entered a clinical trial after promising results in a mouse model [230]. It has been more difficult to find suitable antigens in solid

tumors for both CAR T and NK cells, but recently a clinical trial has opened to treat patients with advanced solid malignancies with NK-92 cells expressing a CAR that recognizes mucin 1 in its cancer-associated glycosylation pattern [229].

Furthermore, it is possible to increase tumor susceptibility to NK cells by engaging activating receptors, such as DNAM-1 and NKG2D, and the death receptor ligand TRAIL through the induction of the expression of their cognate ligands and receptors, respectively, on tumor cells. Radiotherapy, chemotherapy and some pharmacological drugs, such as histone deacetylase inhibitors, cause tumor cell death. The cellular stress responses they induce, including the DNA damage response, upregulate NKG2D and DNAM-1 ligands as well as TRAIL receptors on the surface of tumor cells, resulting in increased lysis by NK cells [82, 231-233]. However, the doses used clinically are not only toxic to tumor cells but also to NK cells. In experimental settings, conventional therapies can be titrated down to low non-cytotoxic doses to achieve anti-tumor immune responses. Given that adjusting dosing of standard therapies is not easily feasible in the clinic, the schedules of conventional therapies and ACT need to be coordinated so as not to damage the infused immune cells.

3.4 RENAL CELL CARCINOMA

There were 338,000 new cases of kidney cancer estimated worldwide in 2012, and 143,000 deaths, making it the 16th deadliest cancer [234]. RCC accounts for 90% of all kidney malignancies and presents most commonly with clear cell histology (70–80%), followed by papillary (10–15%) and chromophobe (3–5%) histologies [235]. Patients with localized tumors that can be surgically removed have a 5-year survival of 70–90%. However, 20–40% of the patients present with metastatic disease at diagnosis and/or experience tumor recurrence after nephrectomy [236, 237], which decreases their survival rates below 20% [238]. Moreover, high Fuhrman grade, which classifies tumors based on their nuclear characteristics, is independently associated with worse survival in patients with clear cell and papillary RCC [239, 240].

The majority of RCC tumors, in particular of clear cell histology, are highly vascular due to marked angiogenesis. In 90% of ccRCC, the von Hippel-Lindau (VHL) protein, which targets the transcription factor hypoxia-inducible factor (HIF) for ubiquitination and proteasomal degradation, is defective [241]. Under hypoxic conditions, often arising in tumors, this results in the accumulation of HIF-induced proteins, including VEGF, EGFR, and platelet-derived growth factor, which mediate angiogenesis and tumorigenesis. In addition, HIF can be activated by mTOR, a master translational regulator of cell growth and metabolism [242]. Consequently, tyrosine kinase inhibitors targeting VEGF and mTOR as well as anti-VEGF

antibodies have become standard of care treatments for advanced RCC, the disease being notoriously resistant to chemotherapy and radiotherapy. However, none of these treatments are curative due to inevitable development of resistance.

Before the arrival of targeted therapies, patients with metastatic disease were mainly treated with IL-2 or IFN-α immunotherapies. Despite toxicities, these therapies have prevailed, for they can confer complete tumor regression and long-term survival to a small number of patients [243, 244]. Indeed, immune cells seem to play an important role in modulating progression of RCC, as suggested by the incidence of spontaneous tumor regressions [17] and high intratumoral NK cell infiltration [245, 246] that has been correlated with better survival in patients with primary and metastatic RCC [131, 247, 248]. In contrast, there has been no beneficial association of T cells with survival in RCC. In addition, circulating and tumor-infiltrating T cells, as well as circulating NK cells, have been found to express high levels of PD-1 [249-251], while RCC tumors have been shown to express PD-L1 [252]. In a recent clinical trial, PD-1 blockade with nivolumab demonstrated improved objective response rates and overall survival compared with an mTOR inhibitor, resulting in its approval for the treatment of metastatic RCC [253]. However, complete responses were achieved in only 1% of the patients, motivating efforts to improve the treatment by combining it with other treatment modalities, of which CTLA-4 blockade and VEGF-targeting therapy seem to be most promising [254, 255].

4 MAIN RESULTS AND DISCUSSION

4.1 STRATEGIES TO IMPROVE NK CELL MIGRATION TO SOLID TUMORS

As the development of ACTs progresses and clinical successes in B cell malignances have led to clinical approvals, the focus is beginning to shift toward targeting solid cancers and addressing one of the key requirements for an effective ACT: lymphocyte homing to tumors. We aimed therefore to develop therapeutic approaches to address inefficient migration to tumor sites by adoptively infused NK cells.

4.1.1 Modulation of chemokines in the tumor microenvironment

The CXCR3/CXCR3 ligand axis has been shown to be important for lymphocyte recruitment and anti-tumor responses in murine models and cancer patients [104, 106, 107, 256]. In **paper I**, we investigated if a locally induced CXCR3 ligand gradient would enhance the infiltration of *ex vivo* expanded human NK cells into tumor-bearing mice in the context of ACT (Figure 2).

Ex vivo expansion of NK cells using high-dose IL-2 and irradiated EBV-transformed LCL feeder cells induced an increase in their expression of CXCR3, conferring them with an improved ability to migrate *in vitro* to recombinant and IFN-γ induced CXCL10 in the supernatant of a number of tumor cell lines. To investigate if CXCL10-mediated infiltration of NK cells would result in an anti-tumor effect, we established melanoma xenograft models in *C.B-17 SCID/beige* and *NOD/SCID/IL2rg*$^{-/-}$ mice. Using *in vivo* imaging, we confirmed that adoptively transferred expanded CXCR3^{+} NK cells preferentially trafficked to CXCL10-overexpressing subcutaneous tumors, resulting in significantly reduced tumor burden and increased survival in mice with CXCL10^{+} tumors compared with those bearing CXCL10^{-} tumors. In a second model, we showed that tumor-derived CXCL10 induced by local IFN-γ

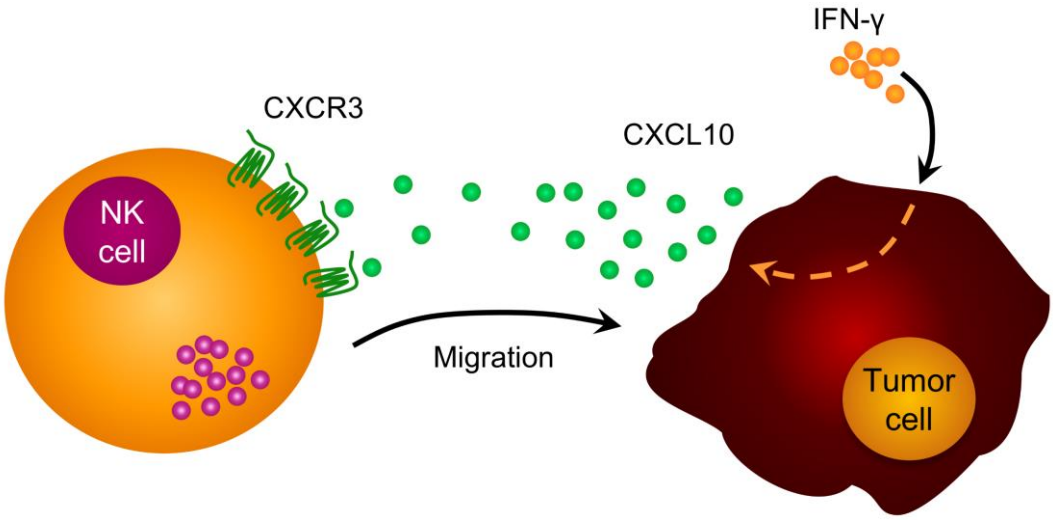

Figure 2. IFN-γ induced secretion of CXCL10 by tumor cells mediates homing of CXCR3^{+} expanded NK cells.

administration increased the influx of adoptively transferred expanded NK cells, resulting in a trend toward lower tumor burden compared with mice not receiving IFN-γ. Notably, the reduction in tumor susceptibility to NK cell lysis due to the IFN-γ-induced increase in HLA class I expression could be counterbalanced by the administration of non-cytotoxic doses of doxorubicin.

Translation of this proof-of-concept study into a clinical setting may pose a challenge. Depending on tumor type, it may not be not possible to get access for a local administration of IFN-γ, and systemic treatment wouldn't only be ineffective but also toxic. However, we could also induce CXCR3 ligands by a combination of IFN-α and poly I:C, both of which are available in the clinic. In addition, localized radiation, epigenetic modulators, locally administered oncolytic viruses, and concomitant anti-PD-1 and ACT treatment have been found to induce CXCR3 ligands in murine tumor models [201, 202, 204, 257]. The IFNs produced by the recruited lymphocytes can, in turn, further upregulate the secretion of CXCR3 ligands by tumor cells or M1 macrophages in the TME, generating an amplification loop that restricts tumor growth. In line with this notion, we observed that CXCL9 and CXCL11 concentrations in the tumors of primary RCC patients positively correlated with the presence of intratumoral $CD8^+$ and total T cells (**paper III**). Since the mouse models we used lack a competent immune system, we were, unfortunately, unable to evaluate if adoptively transferred NK cells would stimulate the influx of host DCs, T cells or NK cells. Nevertheless, our findings emphasize the importance of the CXCR3/CXCR3 ligand axis in directing *ex vivo* expanded NK cells toward solid tumors in order to improve the efficacy of ACTs.

4.1.2 Modulation of the NK cell chemokine receptor repertoire

Solid tumors secrete ligands for the chemokine receptor CXCR2 to promote processes important for tumorigenesis, including angiogenesis, survival, and migration [109]. Adoptive transfer of lymphocytes with high CXCR2 expression is therefore a promising approach to localize them to tumors and improve the efficacy of the treatment. In **paper II**, building on findings with CXCR2-tranduded T cells in melanoma [206, 207], we genetically engineered human NK cells to express CXCR2 to test if this would improve their migration along RCC tumor-derived chemokine gradients (Figure 3).

We detected higher concentrations of CXCR2 ligands in tumors than in plasma in a cohort of 14 patients with primary RCC. However, we observed that healthy donor NK cells expanded *ex vivo* for ACT rapidly downregulated CXCR2 expression. To exploit the CXCR2 ligand gradient therapeutically, we transduced expanded NK cells with human CXCR2 using a retroviral system. Ectopic CXCR2 expression enabled NK cells to specifically migrate toward

recombinant CXCR2 ligands and supernatants from an array of RCC cell lines in transwell migration assays, resulting in increased lysis of K562 target cells. In addition, while their functionality in terms of cytotoxicity, IFN-γ production, and proliferation remained unchanged compared with control NK cells, $CXCR2^+$ NK cells had higher adhesion molecule expression and formed more conjugates with K562 target cells.

Exploiting the chemokine gradient present in the TME by genetically engineering lymphocytes to express the relevant chemokine receptors is an intriguing concept, especially as an addition to a CAR construct. Indeed, overexpression of the chemokine receptors CCR4 and CCR2b has been shown to enhance migration of CAR T cells in xenograft models of Hodgkin's lymphoma and neuroblastoma, respectively [209, 258]. Similarly, CXCR4 overexpression increased homing of the YST NK cell line with an EGFR-targeting CAR in a glioblastoma model [211]. With the strong CXCR2 ligand gradient we detected in RCC patients, the obvious chemokine receptor to transduce into NK cells expanded for ACT was CXCR2. Many other cancers, in addition to RCC, secrete CXCR2 ligands, including lung, ovarian, prostate cancer, and melanoma [109, 206], suggesting that they could also be targeted by this approach.

Endogenous CXCR2 was downregulated on cultured NK cells, probably due to receptor internalization following prolonged exposure to CXCL8 produced by activated NK cells [259]. However, ectopic CXCR2 expression was stable for at least two weeks post transduction, a time frame suitable for the use in preclinical models and in patients, as well as after exposure to CXCR2 ligands. While it may be debated if a stable gene expression justifies the potential risks for insertional mutagenesis and if a transient gene expression achieved by transfection would

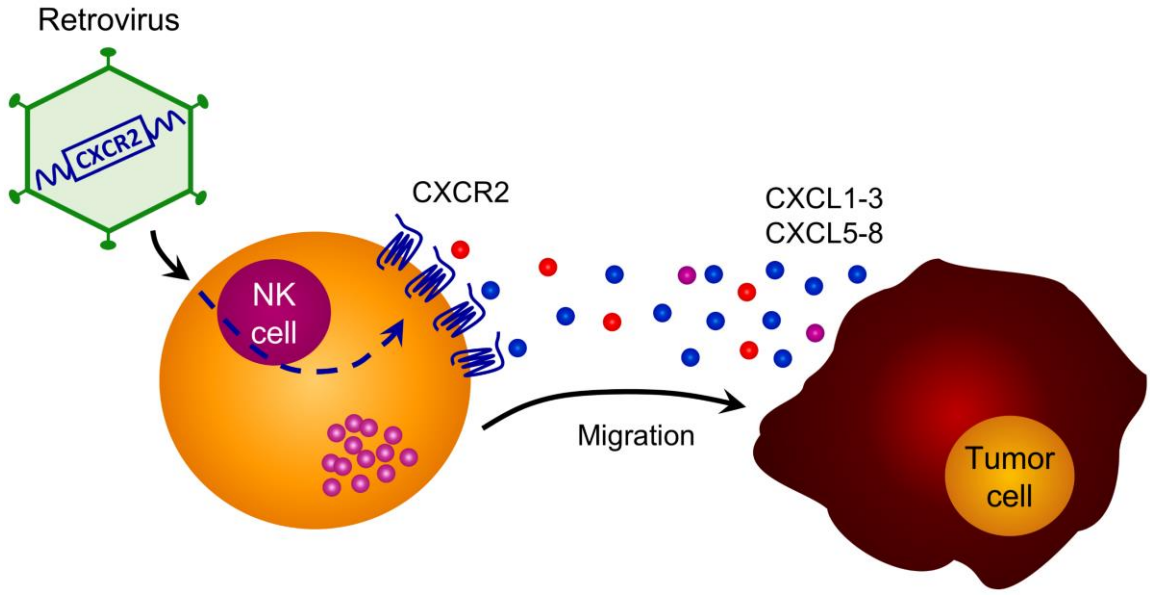

Figure 3. Expanded NK cell transduced with CXCR2 acquire the ability to migrate to tumors producing CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXC7, and CXCL8.

suffice to direct the infused cells to the tumor, the extensive experience using retroviral systems for the transduction of CAR T cells in the clinic has established that this approach is safe [260].

The increased tumor cell lysis of CXCR2-transduced compared with control-transduced NK cells relied on their enhanced migration as their functionality remained unaltered. While our findings prove the concept, studies in mouse models are needed to investigate if CXCR2 transduction confers increased intratumoral accumulation of adoptively transferred NK cells and, most importantly, improves NK cell anti-tumor effects.

4.2 UNDERSTANDING THE NK CELL-ASSOCIATED IMMUNE LANDSCAPE IN RENAL CELL CARCINOMA

Intratumoral NK cell infiltration is positively correlated with survival in patients with primary and metastatic ccRCC [247, 248]. However, immune responses seem ineffective as 20–40% of the patients present with metastatic disease at diagnosis and/or experience tumor recurrence after nephrectomy [236, 237]. In **paper III**, we therefore aimed to identify cellular and soluble factors that mediate NK cell infiltration, activation, and immunosuppression in a cohort of 14 patients with RCC and relate them to clinical parameters.

4.2.1 Mechanisms of immune escape from NK cells

Using multivariate analysis, we identified profound alterations in intratumoral NK cell phenotypes, as well as T cell phenotypes, compared with those in peripheral blood (Figure 4). In addition, we could correlate immune cell markers in the tumor with the Fuhrman nuclear grade and in the blood with the primary tumor stage.

4.2.1.1 DNAM-1 downregulation

We found that high DNAM-1 expression and frequencies of DNAM-1^{+} CD56^{bright} NK cells in RCC tumors were associated with a lower Fuhrman nuclear grade in patients with primary RCC. However, DNAM-1 expression and frequencies of DNAM-1^{+} NK cells, as well as T cells, were profoundly decreased in tumors compared with blood. *In vitro* cultures of PBMCs with RCC cell lines suggested that DNAM-1 downregulation is a tumor-mediated effect dependent on PVR expression levels, and possibly leads to reduced NK cell activation. Consistent with our findings, tumor-associated NK cells in patients with MDS, NSCLC, ovarian, and breast cancer have been shown to have decreased DNAM-1 expression and concomitantly reduced anti-tumor responses [85, 136, 141, 261]. In addition, DNAM-1 expression on NK cells has been reported to inversely correlate with tumor Nectin-2 and PVR expression levels in AML and ovarian cancer, respectively, and to be downregulated in co-cultures with tumor cells

expressing DNAM-1 ligands [136, 146, 262]. Indeed, surface expression of DNAM-1 on T and NK cells seems to be dynamically modulated by *trans*-interactions with PVR, as suggested by elevated DNAM-1 levels in $CD155^{-/-}$ mice [263]. Although the molecular mechanism of DNAM-1 downregulation is not known, it is likely to involve a fast process, such as receptor endocytosis.

We further found in our RCC patient cohort that DNAM-1^{+} $CD56^{dim}$ NK cells in peripheral blood were inversely correlated with frequencies of PMN-MDSCs, and there was a trend for an inverse correlation with iNOS expression levels on PMN-MDSCs. The suppressive effects of PMN-MDSCs on NK cells are poorly studied as most studies have been performed with M-MDSCs or mixed populations. However, PMN-MDSCs have been shown to downregulate NKG2D expression on NK cells in a cell-cell contact dependent manner in a murine breast cancer model [264]. Recently, human M-MDSCs have been reported to express high levels of Nectin-2 and PVR [265]. It is therefore plausible and should be investigated in future studies if PMN-MDSCs in RCC also have high expression of DNAM-1 ligands that can modulate DNAM-1 levels on circulating NK cells.

Collectively, our findings suggest that decreased DNAM-1 expression may facilitate disease progression and present a tumor immune escape mechanism of RCC. In addition to downregulating DNAM-1 expression, DNAM-1 ligands on tumor cells and MDSCs can engage with TIGIT on NK cells and potently suppress NK cell cytotoxicity [92, 265]. Blocking the interactions between TIGIT and PVR has been shown to restore NK cell functionality [92, 225]. However, it represents a challenge to address the reduction in DNAM-1 surface levels. DNAM-1 interactions with cognate ligands are required for NK cell activation, but there may be a threshold above which PVR-mediated DNAM-1 downregulation outweighs the activating signaling. While DNA damage-inducing therapies can upregulate DNAM-1 ligand expression, it seems impossible to control to what degree. Similarly, TIGIT blockade may result in an overabundance of DNAM-1 ligands. Thus, while there may be an optimal DNAM-1 ligand expression level that would induce complete NK cell activation, it will be unlikely achieved in the clinic. However, promising data from mouse models and clinical trials that modulate the DNAM-1/DNAM-1 ligand axis suggest that this may not be a crucial factor for therapeutic efficacy.

4.2.1.2 CD16 downregulation

In addition to DNAM-1, CD16 expression levels and frequencies of $CD16^{+}$ cells among $CD56^{bright}$ and, to a lesser extent, $CD56^{dim}$ NK cells were decreased in tumors compared with peripheral blood. Our findings are corroborated by previous reports that found reduced CD16

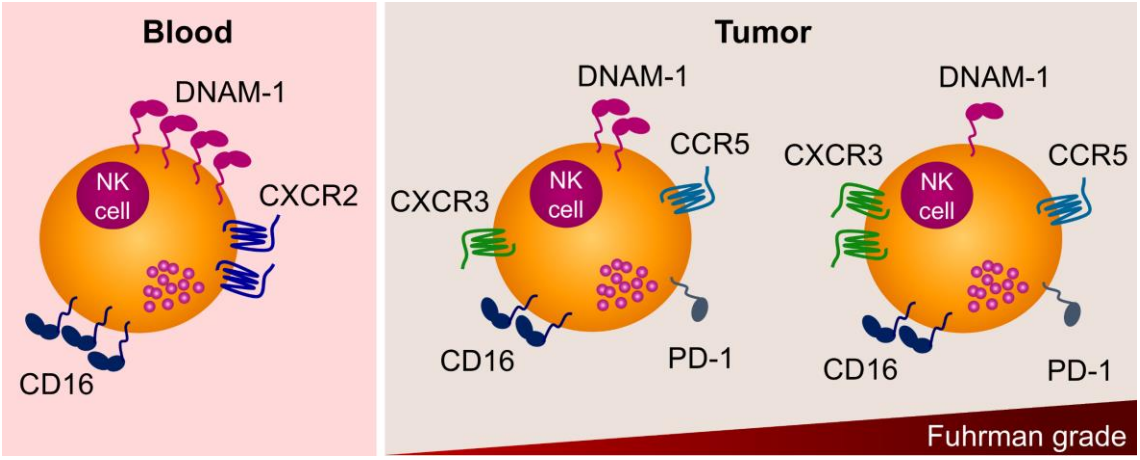

Figure 4. Intratumoral NK cells of renal cell carcinoma patients have profoundly altered phenotypes compared with peripheral blood NK cells, with reduced expression levels of DNAM-1, CD16 and CXCR2, and increased levels of PD-1, CXCR3 and CCR5. Tumors with higher Fuhrman grades are associated with low DNAM-1 and high CXCR3 expression.

expression levels on intratumoral NK cells in ovarian cancer, NSCLC, and CRC [112, 136, 146]. The likely mechanism for the downregulation of CD16 is matrix metalloproteinase (MMP)-mediated shedding, as has been shown in NK cells activated by cytokines and tumor cells [266, 267]. This immune escape strategy by RCC and other tumors implies that antibody-mediated therapies that rely on ADCC by NK cells may be of limited efficacy. Fortunately, inhibition of MMPs, in particular ADAM17, has been found to abrogate CD16 shedding and enhance NK cell cytokine production upon CD16 engagement [266, 267]. An ADAM17/ADAM10 inhibitor has shown clinical benefit in the treatment of $HER2^+$ breast cancer in combination with trastuzumab and is currently in clinical trials in combination with rituximab for the treatment of B cell lymphoma [268]. However, shedding of CD16 by ADAM17 has recently been discovered to be important for NK cell detachment from opsonized targets and their serial killing ability (Daniel M. Davis, presentation at the Scandinavian Society for Immunology Meeting, 2017). These findings raise concerns that pharmacological inhibition of CD16 shedding could compromise strategies based on NK cell-mediated ADCC.

4.2.1.3 PD-1 upregulation

We found that frequencies of $PD\text{-}1^+$ T and NK cells were increased in RCC tumors compared with peripheral blood as well as after *in vitro* cultures with RCC cell lines. Furthermore, PD-1 expression on peripheral blood NK and $CD8^+$ T cells correlated with a lower primary tumor stage and was associated with an activated phenotype of those cells. Induction of PD-1 on NK cells has been observed upon extended exposure to activating cytokines, such as IL-2, or to target cells expressing NCR ligands [269, 270]. In line with our findings, elevated PD-1 expression has been reported on tumor-associated NK cells of patients with ovarian and hepatocellular carcinoma, and on peripheral blood NK cells in patients with MM [144, 145,

269]. In RCC patients, PD-1 expression on tumor-infiltrating lymphocytes has been correlated with poor survival [271, 272]. Persistent PD-1 signaling leads to T and NK cell exhaustion and impaired function. However, PD-1 expression can also be a sign of activation upon tumor encounter. High PD-1 expression on activated circulating lymphocytes in low stage tumors and lower expression in more advanced, but still localized, tumors may be explained by a scenario where T and NK cells re-circulate after contact with and activation by small tumors, while they may encounter exclusion or cell death in larger tumors. This interpretation is supported by a study in RCC patients that included healthy donors and patients with metastatic disease, and found that PD-1 expression was highest in stage 1 and 4 patients, when lymphocytes might have access to smaller lesions (corresponding to metastases in stage 4) [251]. Given that PD-1^{+} T and NK cells seem to be tumor-experienced, but possibly functionally suppressed effector cells, PD-1 blocking therapy before nephrectomy could be worth considering to establish an immune response against tumor recurrence.

4.2.2 Lymphocyte infiltration into RCC tumors

In contrast to many other cancers, there was no accumulation of CD56^{bright} NK cells in RCC tumors compared with peripheral blood, which is in line with a study by Carrega *et al* [112]. We found that CXCR2 ligands were highly enriched in RCC tumors, with CXCL5 expression levels positively correlating with intratumoral CXCR2^{+} NK cells (**paper II**). However, CXCR2 expression and frequencies of CXCR2^{+} CD56^{dim} NK cells were substantially lower in tumors than in peripheral blood (**paper II** and **III**). These findings indicate that CD56^{dim} NK cells infiltrate the RCC tumor microenvironment following a CXCR2 ligand gradient and subsequently internalize the receptor due to chronic exposure to CXCR2 ligands abundant in the TME [273].

Another receptor mediating tumor infiltration appeared to be CCR5 as frequencies of CCR5^{+} T and NK cells, and CCR5 expression levels on these cells, were increased in tumors compared with peripheral blood. In addition, we found a positive correlation of intratumoral T and NK cell frequencies with CCR5 ligands CCL11 and CCL13, respectively (data not shown). CCL5, the most studied CCR5 ligand in lymphocyte migration, was, unfortunately, not included in our chemokine multiplex panel and thus couldn't be evaluated. Several studies have previously described a role for CCR5 in T cell infiltration in tumors of RCC patients [106, 246, 274], while CCR5 involvement in NK cell recruitment in cancer patients had not yet been reported.

The CXCR3/CXCR3 ligand axis was confirmed to be important for T and NK cell homing to RCC tumors. The majority of tumor-infiltrating CD8^{+} T cells expressed CXCR3 and positively

correlated with CXCR3 ligand concentrations in the TME, which is in line with previous reports in RCC [106, 274]. However, frequencies of circulating $CXCR3^+$ $CD8^+$ T cells were higher than found in other studies and decreased in tumors to levels comparable with other studies, possibly through a mechanism of receptor internalization. Although CXCR3 expression and frequencies of $CXCR3^+$ NK cells were higher in tumors than in peripheral blood, NK cell infiltration didn't correlate with concentrations of CXCR3 ligands. This observation could potentially be explained by competition for the same chemokines by both T and NK cells. Mutual regulation of murine T and NK cells has been previously described in the context of immunotherapy, with the deletion of one cell population resulting in the expansion of the other population [275]. Because in our patient cohort T cells expressed higher levels of CXCR3 than NK cells, they could have been at an advantage in migration.

4.3 METHODOLOGICAL CONSIDERATIONS

4.3.1 Xenograft mouse models

Preclinical models are crucial to test new treatments for cancer, and much effort has been put into the development of immunodeficient mouse models to enable engraftment of human tumors. For the evaluation of ACTs using human lymphocytes, it is also important to minimize possible host immune responses. In *C.B-17 SCID/beige* mice, the *scid* mutation impairs the development of mature T and B cells [276], while the *beige* mutation leads to NK cell dysfunction [277]. The *NOD/SCID/IL2rg*$^{-/-}$ mice are a further development of immunodeficient models, for they lack mature T and B cells, functional NK cells and functional cytokine signaling through the common cytokine receptor gamma chain (*IL2rg*), and enable better engraftment of human cells due to the NOD phenotype conferring inhibition of phagocytosis by host macrophages [278]. We have used the described xenograft mouse models to answer specific questions about NK cell migration and for proof of concept (**paper I**). These models preclude the evaluation of NK cell interactions with other components of the immune system in terms of competition for cytokines or suppression by inhibitory cell populations. In contrast, humanized mice reconstituted with a complete human immune system offer the possibility to investigate human NK cells in concert with other factors and cells that influence their activity. Apart from being difficult to establish, the complexity of humanized mouse models might complicate the evaluation of the contribution of particular aspects of the immune response, which, in turn, is the advantage of the mouse models we used.

4.3.2 Flow cytometry analysis

Flow cytometry has been used as an important tool for the analysis of immune cell phenotypes and functions throughout this thesis (**papers I–III**). In addition, flow cytometry was utilized to detect NK cell conjugates with target cells (**paper II**), to determine functionality of the transduced CXCR2 receptor by detecting the release of fluorochrome-bound calcium (**paper II**), and for a more objective quantification of migrated cells in transwell assays (**paper II**), substituting manual counting of hematoxylin-stained cells on transwell membranes (**paper I**).

In particular, we used flow cytometry to characterize immune cell phenotypes on fresh peripheral blood and tumor samples of a RCC patient cohort (**paper III**). We have designed multi-color flow cytometry panels to get a broad overview of NK cells in terms of activation and maturation markers, and, due to our long-standing interest in migration, chemokine receptor expression. Importantly, because of a possible rapid downregulation of chemokine receptors during processing, we analyzed their expression on whole blood samples. Whenever possible, we also analyzed these parameters for T cell populations. Due to their association with worse survival in RCC and known suppressive function on NK cells, panels were designed to evaluate Treg and MDSC populations. The use of fresh samples, although logistically challenging, allowed us to correctly assess frequencies and phenotypes of PMN-MDSC, a cell subset sensitive to cryopreservation [279]. However, we decided against an evaluation of MDSCs in tumor samples due to previous difficulties with their detection in breast cancer samples. Another immunomodulatory cell population abundant in RCC are TAMs [280]. Due to high plasticity of their phenotypes that would need to be captured in multiple flow cytometry panels, we decided to limit our overview analysis to immunohistochemistry, which is yet to be performed. Initially, we planned to characterize tumor cells regarding the expression of ligands for NKG2D and DNAM-1 as well as inhibitory markers. However, the poor viability and difficulties to unambiguously define tumor cells by flow cytometry limited our analysis to a few samples.

The experience with 14 patients has indeed given us insights into how to optimize the study in the future. Undoubtedly, we need to include samples from healthy kidney tissues. With the characterization of tissue-resident NK cells, it has become apparent that their phenotypes are different from circulating NK cells. To dissect tumor-induced alterations on NK cells in RCC tissues, NK cells residing in healthy kidney tissues need to be characterized, which has yet to be reported. Moreover, evaluation of intratumoral MDSC populations should be performed since it was possible to detect MDSCs in one of our tumor samples and in a recently published study [281]. Finally, the flow cytometry panels need to be adjusted to include additional markers of interest that have emerged during the study, such as DNAM-1 family members TIGIT and

CD96, replacing those that have not been informative, such as IFN-γ and TRAIL. This process is already ongoing in MDSC and Treg panels.

Although the newest flow cytometers can detect up to 27 biological markers in one sample, the resolution of complex tissues or cell populations is still limited due to a finite number of suitable fluorescent dyes, overlap of their emission spectra, and autofluorescence [282]. A novel technology that offsets these limitations is mass cytometry or cytometry by time-of-flight (CyTOF). Mass cytometry offers measurement of currently 40 (theoretically up to 100) parameters at single-cell resolution by labeling cells with rare metal-tagged antibodies that together are vaporized into ion clouds and eventually detected based on their mass and velocity [282]. A study analyzing 37 parameters, including 28 NK cell receptors, on peripheral blood NK cells has uncovered an extraordinary degree of NK cell diversity, with a median of 15,000 phenotypic subsets within an individual [283]. Recently, a CyTOF analysis of tumor biopsies from patients with ccRCC has detected 11 $CD8^+$ T cell phenotypes, 8 $CD4^+$ T cell phenotypes, and 17 TAM phenotypes, with heterogenous expression patterns of suppressive markers [284]. However, mass cytometry comes with its own limitations. For instance, CyTOF loses 50–70% of the cells in a sample [282], thus requiring a large amount of material to measure rare events. A crucial bottleneck in the analysis of patient material, however, is its limited amount. Nevertheless, these studies demonstrate that mass cytometry is a powerful tool to characterize the complex network of immune cells in blood and TME, which could not only be used for the discovery of prognostic and predictive markers but also novel therapeutic targets.

4.3.3 Multivariate statistical analysis

The analysis of a 'short-and-wide' data set with a small number of study subjects and many measured variables represents a challenge for univariate statistical tools because of a high number of potential false positives that can be generated by repeated hypothesis testing. The methods applied to correct for false positives also reduce statistical power, thereby removing true positives [285]. In addition, univariate approaches assume that each variable is independent. In a biological system, however, there is a high degree of interdependency or multi-collinearity. For these reasons, multivariate statistical analysis tools were utilized in the analysis of RCC patient data (**paper III**).

We applied the supervised method Orthogonal Projections to Latent Structures (OPLS) to find correlations between clinical parameters, and the variables measured in blood and tumors by flow cytometry and multiplex analysis, including cell population frequencies, expression levels, and cytokine concentrations. One advantage of OPLS is that it separates systematic variation in the data into a predictive component correlated to Y, in our case the Fuhrman nuclear grade

or the primary tumor stage, and an orthogonal or unrelated component [286]. This allows for selection of biomarkers of disease progression. To determine differences in immune signatures between blood and tumor samples, we used the recently developed tool OPLS-Effect Projections (OPLS-EP) that takes into account a matched or paired sample setup [287]. This method requires to create an 'effect matrix' containing each variable by means of subtraction. While this renders the visual interpretation less intuitive, so far there seem to be no other outstanding tools in multivariate statistics to analyze paired sample structures.

The generated models greatly depend on the quality of the input data. There are model quality parameters that need to be carefully considered when making interpretations. In our data set 16% of the data were missing; however, OPLS can cope with moderate amounts of missing data [286]. In addition, our patient cohort was skewed toward women; however, the gender variable did not significantly influence the calculated models. Nonetheless, blood samples from healthy donors and blood and tumor samples from RCC patients with metastatic disease should be included in future for a better identification of biomarkers for disease progression and/or recurrence.

5 CONCLUSIONS AND OUTLOOK

"The way we treat cancer is about to change forever."

– Neil Canavan, A Cure Within, 2018

Cancer immunotherapy has become the fifth pillar of cancer treatment, joining the ranks of surgery, radiation, chemotherapy, and targeted therapies. Developments in immune-oncology over recent decades have brought unprecedented clinical benefits to patients. However, to truly cure cancer or turn it into a chronic disease with the help of the immune system still requires further research on a basic as well as applied level.

5.1 CONCLUDING REMARKS

This thesis provides proof of concept for two approaches to improve the efficacy of adoptively transferred NK cells against solid tumors by promoting their trafficking to tumor sites. In addition, it identifies a tumor immune escape mechanism and potential biomarkers of disease progression in patients with primary RCC. The major conclusions from the present work are as follows:

- Locally induced CXCL10 in melanoma tumors enhances infiltration and anti-tumor responses of adoptively transferred $CXCR3^+$ human NK cells (**paper I**).
- Genetic engineereing of human NK cells to express CXCR2 augments their migration along recombinant and RCC-derived CXCR2 ligand gradients, enhancing target cell killing (**paper II**).
- NK cells, as well as T cells, are profoundly altered by the tumor microenvironment in primary RCC patients, with downregulation of DNAM-1 expression representing a mechanism of immune escape (**paper III**).
- Low DNAM-1 expression on intratumoral NK cells and low PD-1 expression on peripheral blood NK and $CD8^+$ T cells are potential biomarkers of disease progression in patients with localized RCC tumors (**paper III**).

Together, these findings advance the rapidly evolving field of NK cell-based immunotherapy of solid tumors and deepen the understanding of NK cell biology in RCC. Migration of adoptively transferred NK cells, while initially neglected over efforts to improve persistence and cytotoxicity, is beginning to draw attention. The presented strategies to enhance NK cell

homing could increase efficacy of NK cell- as well as T cell-based cell therapies when translated into the clinic. Moreover, the implications of tumor-induced changes of NK cells in RCC require further investigation, complemented by functional studies. In particular, the role of DNAM-1 family members should be explored in tumor immune escape in RCC. In addition, the contribution of NK cells in patients responding to PD-1 blockade needs to be elucidated.

5.2 FUTURE DIRECTIONS – THE X FACTOR

Treatment with immune checkpoint inhibitors has undoubtedly been the greatest breakthrough in cancer therapy 'raising the tail' of the survival curve, particularly in melanoma patients. However, owing to the complexity of immunoregulatory systems and the heterogeneous nature of different cancer types, tumor genetics and epigenetics, as well as factors related to host immunity, there is a need for more efficient immunotherapy regimens, combining, as Emens *et al.* put it, PD-1 blockade + CTLA-4 blockade + 'X' [288]. Indeed, over 1,100 clinical trials are currently evaluating the combination of anti-PD-1/PD-L1 agents with other therapies, predominantly anti-CTLA-4 antibodies and chemotherapies [151]. But could NK cells be the 'X' factor in the equation?

While NK cells are generally regarded to be ineffective against established solid tumors, clinical trials in neuroblastoma and NSCLC have successfully induced NK cell responses, resulting in encouraging patient outcomes [289-291]. Importantly, NK cells could step in as effector cells when tumors develop resistance to T cell attack facilitated by PD-1 blockade through disruption of antigen presentation or MHC class I expression [292]. In addition, the ability of NK cells to target metastasizing tumor cells as well as CSC-like cells resistant to standard therapies [125] holds great promise for NK cell therapies in the context of residual disease after tumor debulking. Considering the induction of PD-1 expression on NK cells in cancer, inhibition of the PD-1 pathway could be an effective tool to unleash cytokine- and tumor-activated NK cells. As described in chapter 3.3.2.4, NK cells have other immune checkpoints — many of them overlapping with T cells — and agents targeting those are currently tested in combination with PD-1 blockade in clinical trials [293]. In light of the DNAM-1 downregulation on lymphocytes in the TME observed in a number of cancer types and described in this thesis, it will be interesting to see the therapeutic effect of anti-TIGIT antibodies that are currently evaluated together with anti-PD-1 and anti-PD-L1 antibodies in patients with advanced solid tumors. Furthermore, it is important to note that NK cells don't only kill tumor cells but also participate in complex interactions with other leukocytes, recruiting and activating APCs and promoting Th1 type responses. Such indirect mechanisms of the NK cell anti-tumor immunity are also important elements of cancer immunotherapy.

6 ACKNOWLEDGEMENTS

Many fantastic people have accompanied me on my way to the PhD, and their support helped me reach this stage.

Andreas, it has been a privilege to have you as the main supervisor for my PhD studies. Thank you for your encouragement and for opening up so many opportunities for me to become an independent scientist. You have been invaluable for my development, I will always be grateful.

My co-supervisors: **Maarten**, you have been my cheerleader even before you became my co-supervisor. I admire your enthusiasm and passion for science, your crazy ideas, and your versatile scientific and non-scientific interests. I am grateful to have you as a friend, and for all the adventures we shared. **Charlotte**, thank you for our discussions, your expertise in mouse studies, and sharing resources from your lab. **Kalle**, my former co-supervisor, you are a visionary. Even from afar your enthusiasm for NK cell research has been inspirational to me. **Rolf**, although never officially my co-supervisor, I am thankful for you sharing your vast scientific experience, fun stories from around the world, and the enthusiasm for the Stockholm archipelago.

The very first PhD students and former members of the Lundqvist group: **Erik**, thank you for setting me off on the way to improve NK cell therapies, and the fun in the lab and during our jam sessions. **Dhifaf**, your tremendous dedication and impressive scientific skills have been a real inspiration to me. I have no doubts about your academic success.

My students: **Christina**, thank you for your hard work, especially on the clinical samples; it wouldn't have been possible without your commitment. **Rosa**, it was fun working with you as you learned quickly and were well organized. Thank you for all the help. **Annet**, your independence and 'never give up' attitude have been impressive – that's the way to go. Thank you for the last-minute help with the revisions. Thanks to all of you for teaching me to be a better mentor.

Current and previous members of the immune-oncology society at CCK a.k.a. the **Lundqvist and Kiessling groups**: **Chen Ziqing** and **Neo Shiyong**, as fresh and almost PhD students, thank you for advancing the NK cell research in the Lundqvist lab. Thanks also to **Caroline**, **Aline**, **Hannah**, **Ann-Kathrin**, and **Kevin**, previous students in the Lundqvist group, as well as new members, **Yang Ying** and **Despina**, for joining our efforts to improve NK cell therapies. **Yago**, thank you for enriching my knowledge not only about flow cytometry but also movies, politics, foreign cuisines, and so much more. **Mao**, you're one of the brightest people I know. Thank you for the scientific discussions and the amazing introduction to China. **Jeroen**, thank

you for your critical questions and scientific discussions. **Ulrika**, I have really enjoyed your direct and open nature, and your honey. **Stina**, you are a fighter, and you will conquer that mountain of work. **Tanja** and **Maria Nyström**, you were the heart of our fika culture that I enjoyed so much. **Yuya**, thank you for the insights into the Japanese culture. **Isabel**, I am glad you have been a frequent guest at CCK, so we could have discussions about science and beyond. Thanks to past and present students in the Kiessling group that have not only made great scientific contributions, but also added so much fun and life to the lab: **Madhura**, **Özcan**, **Giusy**, **Bettina**, **Iva**, **Maxi-Lu**, **Jochen**, **Laura**, **Roeltje**, **Felix**, **Ernesto**, **Bobby**, **Frida**, **Eirini**, **Jan**, **Disha**, **Aine**, **Maria Wolodarski**, and **Linus**.

The people that help run the place: **Elle** and **Elisabeth**, thank you. **Sören**, thank you for making my workplace as ergonomic as possible.

Much of the work wouldn't have been possible without collaborators: **Kenth**, your skills with mice saved my day on many occasions. **Ann-Helén Scherman-Plogell**, thank you for providing the RCC patient samples and letting us witness a robotic surgery. **Ulrika Harmenberg**, I really appreciated the opportunity to join you to see patients. **Elina**, thank you for your patience and optimism when introducing me to SIMCA. **Sanela**, our discussions about SIMCA gave me exactly the right input for the study. **Nick**, I am grateful for your help with bioinformatics, and your professionalism and patience in our discussions. **Valentina,** your commitment to science is admirable. Thank you for sharing your data. **Mike Nishimura**, I am so grateful that you took me into your lab at the Loyola University Chicago and had your fantastic group members **Gina**, **Dave**, and **Patricia** teach me valuable techniques, in addition to giving me an incredible introduction to the American culture. **Bruce Freimark** at Peregrine Pharmaceuticals, thank you for the opportunity to collaborate with you. The **administration and researchers at the Center for Collaborative Research** at Nova Southeastern University, thank you for welcoming me in your Visiting Research Scholar program and the great scientific and cultural exchange.

I am grateful to have been part of many activities at CCK and KI, and to have met many great people to share discussions, coffee, rooms, laughter, excitement, difficulties, protocols, and cooking recipes. **Ahmed**, you asked me to join the organizing team for 'Nov2K 2014', opening the door to one of the most remarkable experiences of my PhD. **Matheus**, thank you for your optimistic 'can do' mentality, which I especially valued during all the projects we have done together. **Gonçalo**, thank you for introducing me to the fantastic 'Winter Conference' and bringing me aboard the team. **Nina**, it's been great fun having you as my conference buddy, even beyond your days at CCK. **Majken**, thank you for keeping talking Swedish to me when I

barely knew the language. **Katja**, **Olga**, **Sue-Li**, and **Smaranda**, thank you for an open ear for my worries. **Tatjana**, **Margarita**, **Linnea**, **Cinzia**, **Anders**, **Anderson**, **Barbro**, **Li-Sophie**, **Ann**, and all the others on the **1st floor of CCK**, thank you for the fikas and creating a great atmosphere. **Emarndeena** and **Sophia**, I really enjoyed working with you on 'OPUS' and getting creative. **Teresa**, I am very glad that we reconnected over the work at 'Medicor'. I admire your strength and dedication. **Tiago**, you are reaching for the stars and always have a plan; this inspires me to strive for more as well. **David**, thank you for never getting tired of fixing my English and a healthy outside perspective on my PhD life.

I am grateful for my friends outside KI that support me and help me have a life outside the lab: **Susann**, **Annika**, **Malte**, **Anna**, **Sim**, **Markus**, **Sara**, **Iván**, **Jenny, Matthieu,** and **Bleuenn** in Sweden, and **Tim** and **Flo** in Germany.

Mama und **Papa**, ich danke euch von ganzem Herzen für eure bedingungslose Unterstützung während meiner Doktorarbeit und im Leben überhaupt. **Баба Надя**, **баба Нина**, наконец-то моя учёба закончилась и начинается 'настоящая' жизнь. Моя работа, к сожалению, уже не вернёт деду Колю и деду Володю, но, надеюсь, она когда-нибудь поможет другим раковым больным. **Vincent's family**, thank you for receiving me so warmly in Belgium and making me feel at home.

Vincent, there are no words to describe how much you mean to me. Your support in all aspects of my life has made it possible for me to get this far. You are my light, my everything.

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